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# UREA CLEARANCE AND DIURESIS IN MAN

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The ratio of the rate of excretion of urea to the concentration of urea in the plasma, that is, the urea clearance, has been known for many years to increase with the flow of urine (1). The quantitative relation between the urea clearance and urine flow has been represented by numerous equations, each adequate for restricted ranges of urine flow. The most common of these equations, in this country, at least, have been summarized in Table I.

However, there is no justification for these multiple equations, except, perhaps, their simplicity and the ease with which their constants can be computed. Actually, when the clearances are plotted on a diagram, the trend indicated by the points follows a continuous curve, without angles or kinks. Therefore the relation between the clearance and urine flow should be represented, mathematically, by a continuous function of the urine flow, with a continuously turning tangent from the beginning to the end. The curve of the clearances resulting from the equations given in Table I does not have this property, since the slope of the curve at the end of one restricted range of diuresis is not equal to the slope of the curve at the beginning of the next. Thus, in passing from the first straight line (Equation 1, Table I) to the square root curve (Equation 2, Table I), the slope changes abruptly at  $v=0.35$  cc. per minute, just as it changes also abruptly at  $v=2$  in passing from the square root curve (Equation 2,

Table I) to the other segment of straight line (Equation 3, Table I). Besides, since the constants given by these equations do not all have the dimensions of a clearance, it is confusing to call them clearances. And if, in order to overcome this difficulty, the first constant is ignored and the remaining two are interpreted as ordinates at selected urine flows, this method transforms the whole curve into two discontinuous clusters of points, one at 1 cc. per minute, the other at 2 cc. per minute.

To these difficulties we may add that Equations 2 and 3 break down altogether in some forms of renal disease—nitrogen retention phase of prostatic obstruction (4)—and in other cases that we shall present here. It is not profitable to increase the number of equations to suit every new case.

This paper will deal with an analysis of the data on the excretion of urea in 4 subjects, 1 normal and 3 nephrosclerotics, data which cover a wide range of excretory function. From this analysis, we shall develop an equation that overcomes the inherent defects in multiple representations. With the help of this equation, we shall be able to follow the changes in the clearance curve in renal disease, and to discuss the concentration ratio of urea.

After presenting the data, we shall therefore consider, specifically, (1) the representation of the data on urea clearance, at all urine flows, by

TABLE I  
Equations representing the urea clearance at various ranges of diuresis

Range of urine flow cc. per minute	Equation	Designation of constant	Dimensions of constant	Geometrical interpretation of constant
$0 < v < 0.35$	1. $C = pv$	p, minimal clearance (2)	pure number	Slope in (C, v) coordinates
$0.35 < v < 2.00$	2. $C = s\sqrt{v}$	s, standard clearance (3)	square root of flow	Slope in (C, $\sqrt{v}$ ) coordinates or ordinate at $v = 1$ in either (C, v) or (C, $\sqrt{v}$ ) coordinates
$2.00 < v$	3. $C = m$	m, maximum clearance (3)	flow	Ordinate at any $v > 2.00$ in either (C, v) or (C, $\sqrt{v}$ ) coordinates

In this table, C represents the clearance and v represents urine flow. Both C and v have the dimensions of flow.



a single equation; (2) the rejection of the concept of a maximum clearance; (3) the changes in the constants of the single equation in renal disease; (4) an objection to the current method of expressing the clearance of a renal patient in per cent of a normal clearance; (5) the concentration ratio of urea, its continuous dependence on urine flow, and its equivalence to the clearance at all urine flows; (6) the value of the limiting concentration ratio, as the urine flow approaches zero, in estimates of renal function; (7) the fallacy of comparing the specific gravity of the urine at small urine flows with the urea clearance at any urine flow; and (8) certain *a priori* considerations that suggest why the clearance of urea and the clearance of other substances should rise with increasing urine flows.

#### EXPERIMENTAL DATA

The cases can be briefly summarized as follows: *Case 1*, a 26-year-old, white woman with normal renal function; *Case 2*, a 38-year-old, white woman with advanced nephrosclerosis, who died shortly after the experiment; *Case 3*, a 45-year-old, white woman with hypertension, in whom a renal denervation was done according to the technique of Peet (5); and *Case 4*, a 44-year-old, white man with progressive nephrosclerosis, in whom a bilateral perivascular sympathectomy of the renal pedicle was done. *Cases 3* and *4* are part of a separate investigation of the renal excretory function of hypertensive subjects, both before and after renal denervation. The complete data of these cases will be presented in another publication. For this paper, we have selected, in *Case 3*, two experiments, 1 and 2 years, respectively, after denervation, and in *Case 4*, one experiment, 21 months after denervation.<sup>1</sup>

The data were obtained in the course of experiments in which not only the excretion of urea, but also the excretion of exogenous creatinine, and, in one case, the excretion of xylose, were investigated. In this paper, only the data on urea excretion will be presented.

The rate of excretion of urea and the plasma concentration of urea were followed in each experiment for 8 to 10 consecutive hours. The blood samples and the urine were obtained at intervals of about 1 hour each, but when diuresis was large the interval of urine collection was naturally shortened. The urea was analyzed by the aeration method of Van Slyke and Cullen (6).

In order to save space, the data are presented graph-

ically in Figures 1 and 2, where the cases are identified by means of subscripts. The urea nitrogen concentration of the urine ( $u$ ) and the urea nitrogen concentration in the plasma ( $x$ ) were calculated in mgm. per cc. of urine and plasma, respectively. The urine flow, or diuresis, ( $v$ ) is given in cc. per minute. The rate of excretion ( $y$ ) is obtained by multiplying  $u$  and  $v$ . The mean rate of excretion ( $y$ ), in mgm. per minute, is assumed to occur at the middle of the interval of urine collection. The plasma concentration corresponding to the middle of this interval ( $x$ ) is obtained by graphic linear interpolation in a diagram in which the plasma concentration is plotted against the time. With these symbols, the urea clearance ( $C$ ) is given by the ratio  $y/x$  in cc. per minute.

As can be seen in Figures 1 and 2, the trend in the clearance in all the 4 cases is always upward, before as well as after a urine flow of 2 cc. per minute, the so-called augmentation limit of Möller, McIntosh and Van Slyke (3). The initial rise is quite steep in *Cases 1* and *3*, low in *Case 4*, and very low in *Case 2*. At no point is the clearance (the mean clearance) independent of diuresis, so that the clearance at a given time cannot be completely defined unless the urine flow at that time is also specified.

#### SINGLE EQUATION

An equation previously proposed (7)

$$C = A(1 - e^{-kv}), \quad \text{Equation 4}$$

in which  $A$  and  $k$  are constants and  $e$  is the base of the natural logarithms, has been shown to fit, at ordinary diuresis, a large set of data (3, 8) better than Equation 2, Table I.

At very low diuresis, it reduces to

$$C = Ak \cdot v, \quad \text{Equation 5}$$

as can be seen by expanding the right hand side of Equation 4 in powers of  $v$  and retaining only the terms to the first power. Equation 4 contains the 3 equations of Table I, as has been recognized by Van Slyke (9). In point of history, it anticipated the experimental data of Chesley (2). But Equation 4, developed as it was for data in which the clearance could be assumed constant at a diuresis beyond 2 cc. per minute, will not satisfy our present data.

However, if we add to it a linear term in diuresis,  $bv$ , thus,

$$C = A(1 - e^{-kv}) + bv, \quad \text{Equation 6}$$

we will get an expression that, at large diuresis, becomes asymptotically equal to the straight line,

$$C = A + bv. \quad \text{Equation 7}$$

<sup>1</sup> The surgical operation of *Case 3* was performed by Dr. Spencer Braden, Visitant Surgeon in charge of Neurosurgery at St. Luke's Hospital, and that of *Case 4* by Dr. Carl H. Lenhart, Professor of Surgery of the School of Medicine, Western Reserve University, then Director of the Surgical Division of St. Luke's Hospital.

# UREA CLEARANCE AND DIURESIS

The curve represented by Equation 6 has a continuously turning tangent, since, from the equation of its first derivative,

$$\frac{dC}{dv} = Ake^{-kv} + b,$$

the slope of the tangent diminishes continuously from the finite value  $Ak + b$  at zero diuresis to the constant value  $b$  at large diuresis. Likewise, the proportionality of the clearance to diuresis, at small diuresis, is preserved, since Equation 6 reduces to the equation

$$C = (Ak + b)v, \quad \text{Equation 8}$$

at very low diuresis. Incidentally, the coefficient  $(Ak + b)$ , Equation 8, which represents the slope of the clearance curve at zero diuresis, will be shown, later in the paper, to be equal to the limiting concentration ratio of urea.

## APPLICATION AND RESULTS

Equation 6 was applied to our data as follows: first, the linear part (Equation 7) was fitted by least squares to all data at  $v$  equal to or larger than 2 cc. per minute; then, using the values of  $A$  and  $b$  thus determined, rearranging Equation 6, and taking the logarithms, we computed a mean value of  $k$  from the equation

$$-k \cdot \log e = \frac{1}{v} \log \frac{A + bv - C}{A}.$$

In this equation  $C$  and  $v$  either assume the values of the clearance at  $v$  less than 2 cc. per minute and their respective urine flows, or represent means of conveniently grouped data. For the purpose of this paper, no further refinement in fitting was considered necessary. The results are shown in Figures 1 and 2.

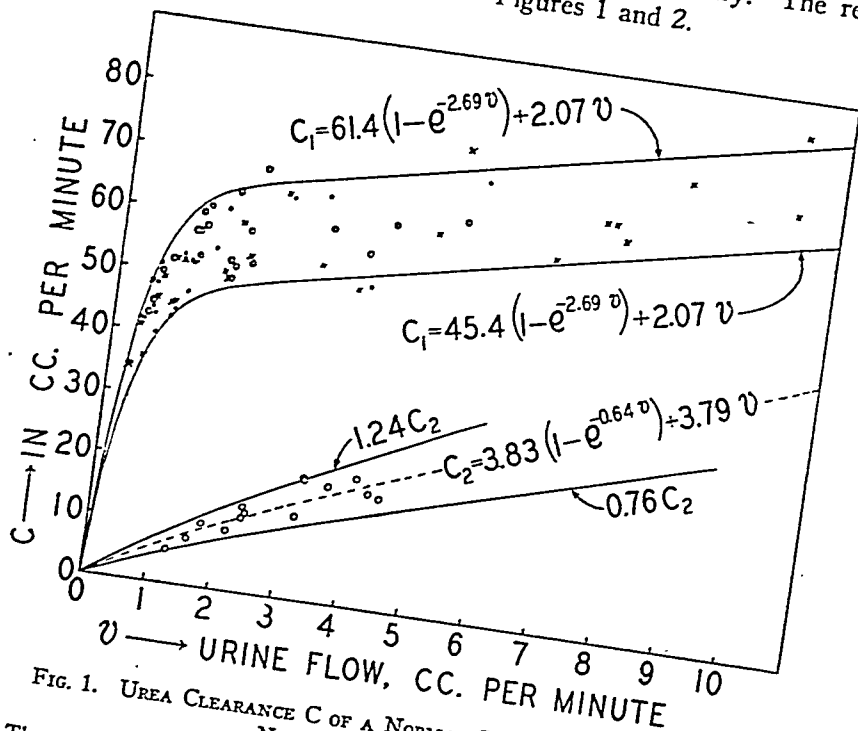


FIG. 1. UREA CLEARANCE  $C$  OF A NORMAL SUBJECT ( $C_1$ ) AND OF A NEPHROSCLEROTIC ( $C_2$ )

The equation for the grouped data of Case 1 is  $C_1 = 53.4(1 - e^{-2.69v}) + 2.07v$ . The points represent endogenous urea data in oral creatinine experiments; the open circles represent urea data following urea and creatinine ingestion; and the crosses represent endogenous urea data in oral xylose experiments. The two curves  $C_1$ , obtained by changing  $A$  by  $\pm 15$  per cent of its mean value, inclose all but 6 of the 68 observations. The open circles of Case 2 represent endogenous urea data obtained during an experiment with ingested creatinine. As shown by the solid curves, a 24 per cent variation of the mean clearance  $C_2$  is needed to include 13 of the 14 observations.

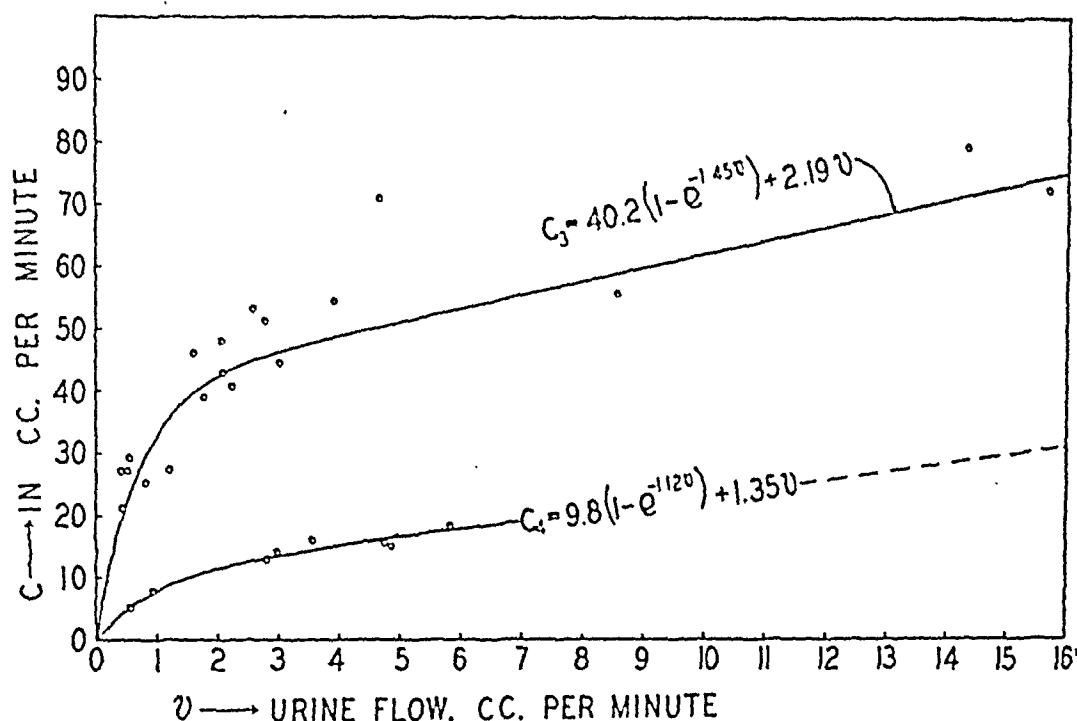


FIG. 2. UREA CLEARANCE ( $C$ ) OF 2 NEPHROSCLEROTICS, ONE ( $C_3$ ) AFTER SPLANCHNICECTOMY AND ANOTHER ( $C_4$ ) AFTER DENERVATION OF THE RENAL PEDICLES

The curve  $C_3$  represents the mean curve for all the data (51 observations) on this subject. The mean position of the points shown is somewhat higher than the mean curve  $C_3$ . The erratic point at  $v = 4.7$  cc. per minute corresponds to a period of sudden increase in urine flow after a long interval of decreasing urine flows. In this subject, a 34 per cent variation of the mean value of  $A$ , 40.2, has to be allowed in order to include 90 per cent of the observations.

The mean values of the constants  $A$ ,  $k$ ,  $b$ , and the standard error of  $b$ , are: *Case 1*,  $A = 53.4$ ,  $k = 2.69$ ,  $b = 2.07$  (standard error 0.548); *Case 2*,  $A = 3.83$ ,  $k = 0.64$ ,  $b = 3.79$  (standard error 0.586); *Case 3*,  $A = 40.2$ ,  $k = 1.45$ ,  $b = 2.19$  (standard error 0.432); and *Case 4*,  $A = 9.8$ ,  $k = 1.12$ ,  $b = 1.35$  (standard error 0.435). By comparing  $b$  with its standard error, and by taking into consideration the number of observations, it will be seen that the slope  $b$  is in each instance significantly different from zero.

This result in the normal subject, *Case 1*, is contrary to the experimental evidence of Möller, McIntosh and Van Slyke (3) in 6 normal subjects, but is in agreement with the experimental data of Chasis and Smith (10) in 9 normal subjects, although Chasis and Smith interpreted their results differently.<sup>2</sup> Our results in nephrosclerosis, *Cases*

2, 3, and 4, and the results of Hayman, Longley and Bobey (4) in prostatic obstruction, together with the results in our normal subject and with the interpretation we give to the data of Chasis and Smith in normal subjects, show that the concept of a maximum clearance does not have the generality that we have been so far led to believe.

The trend and scatter of the points in our ~~cases~~ require a few comments. In *Case 1*, the scatter of the points is quite large, but at flows larger than 2 cc. per minute the points seem to lie within a

high value." These authors did not determine the mean slope of the linear part of their data, but by means of a stretched thread we estimate these slopes to be 1.14 for their Figure 1 and 1.5 for their Figure 2. Seeing that the scatter of their points is less than in our normal subject, *Case 1*, and that the range of urine flows is larger, we hazard the suggestion that, had their data been subjected to statistical treatment, the slope of their data at large diuresis would have proved significantly different from zero. That Chasis and Smith, however, did not believe this to be the case, is evident from their statement [(10) page 357]: "Inspection of Figures 1 and 2 shows that our data conform roughly to the 'standard' and 'maximum' clearance concept of Möller, McIntosh and Van Slyke."

<sup>2</sup> Chasis and Smith describe the urea clearances illustrated by them as follows [(10) page 350]: "Inspection of the absolute urea clearance, as portrayed in Figures 1 and 2, shows that there is a progressive increase in the urea clearance as the urine flow increases from low to

zone bounded by parallel lines, as if most of the variation could be charged to  $A$  rather than to  $b$ . By adding to  $A$ ,  $\pm 15$  per cent of its mean value, 53.4, we get the two lines shown, inclosing 91 per cent of the points (Figure 1).

In *Case 2*, the trend of the observations is well represented by the curve  $C_2$ , Figure 1. Although the absolute variation is smaller than in *Case 1*, the relative variation is larger. In fact, in order to inclose practically all the data, it was found necessary to vary  $C_2$  by 24 per cent. But, since  $A$  and  $b$  have about the same numerical magnitude, we cannot lay all the variation on  $A$ .

In *Case 3* the scatter of the data is very great. Although a detailed account of the data on this case will be published later, it may now be said that the curve  $C_3$  represents the mean curve fitting all the data in this subject. A variation of 34 per cent of the mean value of  $A$ , 40.2, is necessary to inclose 90 per cent of the observations. The large diureses recorded were neither induced nor controlled by the conditions of the experiments. Had we controlled diuresis, as Chasis and Smith did (10), we would very likely have lessened this scatter. The erratic point at  $v=4.7$  corresponds to a sudden increase in diuresis interrupting a rather long interval of slowly decreasing diuresis.

In *Case 4* the scatter is least. Yet, the constant  $b$  in this case, 1.35, is the least well determined, partly because of its small magnitude, partly because of the small number of observations.

It may be of interest to notice that the persistent effect of urine flow on the clearance occurs in both the normal kidney (*Case 1*) and the arteriosclerotic kidney (*Cases 2, 3 and 4*), and in the latter not only when the nerve supply of the organ is intact (*Case 2*), but also when it has been severed (*Cases 3 and 4*).

#### EFFECT OF RENAL DISEASE ON THE CONSTANTS

Comparison of the constants in these cases shows that in renal disease the constants  $A$ ,  $k$ , and  $b$ , are not affected to the same extent. For instance,  $A_2$  is only about 7 per cent of  $A_1$ ,  $k_2$  about 24 per cent of  $k_1$ , and  $b_2$  actually larger than  $b_1$ . This unequal effect of disease on the constants is of considerable practical significance. To be sure, the constant  $A$  may diminish in all types of renal disease, as is generally known, but  $k$  also may

diminish, and, when  $k$  does diminish, the shape of the curve is no longer similar, in the mathematical sense, to its former shape, irrespective of any change in  $b$ . After careful examination of the "atypical clearances" in prostatic obstruction, illustrated by Hayman, Longley and Bobey (4), we believe that all of them could be shown to be particular examples of Equation 6, on a par with the clearances of our nephrosclerotics.

The extreme case of a clearance rising linearly with urine flow can be arrived at theoretically in the following way. If the kidney loses all power to concentrate urea and yet excretes urine, the rate of excretion of urea,  $y$ , would be

$$y = v \cdot u = v \cdot x,$$

or, since  $C = y/x$ ,

$$C = v.$$

In other words, the smallest clearance compatible with a urine flow greater than zero is that in which the clearance is equal to the urine flow itself, and obviously it could not be less than this, except in anuria. This extreme clearance, which is represented by a straight line with slope 1, can be obtained directly from Equation 6 by making either  $A$  or  $k$  equal to zero, and  $b$  equal to 1.

#### CRITICISM OF PER CENT COMPARISON OF UREA CLEARANCES

By writing out the ratio of two clearances, using for each the form given by Equation 6, we can see that no two clearances can be proportional to each other unless the constant  $k$  of one is equal to the constant  $k$  of the other, and unless  $b$  changes proportionately with  $A$  in one of them or is equal to zero in both. But, we have just shown that in renal disease  $k$  changes, and  $b$  is neither equal to zero nor does it decrease proportionately to  $A$ ; consequently, the practice of expressing a patient's clearance in per cent of a normal clearance is, in general, not justified. For example, the ratio of the clearances ( $C_2/C_1$ ) at diuresis of 1, 2, 3, and 4 cc. per minute is, respectively, 0.108, 0.181, 0.246, and 0.303. We cannot conclude from this that the clearance of *Case 2* lies somewhere between 11 and 30 per cent of that of *Case 1*, because this conclusion would be at once vague and misleading. If we say that the clearance of *Case 2* is 11 per

cent of the normal at ordinary diuresis, we ignore the effect of large diuresis. If, on the other hand, we conclude that, at a diuresis of 4 cc. per minute, *Case 2* will reach a clearance as large as one-third that of the normal, we will shift the emphasis from the impairment of the kidney to the possible beneficial effect of large diuresis.

We must look for another way to compare the degree of renal impairment. The one that presents itself is the limiting concentration ratio of urea, a constant independent of urine flow, as will be shown shortly.

#### CONCENTRATION RATIO OF UREA

The concentration ratio is the ratio of the concentration in the urine,  $u$ , to the concentration in the blood plasma,  $x$ . Since the rate of excretion of urea,  $y$ , is determined by multiplying  $v$ , the urine flow, by  $u$ , the urea concentration in the urine, the equation of the concentration ratio can be obtained from that of the clearance by dividing both members of the latter equation by the urine flow. In general, therefore, if the clearance is represented by any given function of diuresis,  $C = f(v)$ , it follows necessarily that

$$\frac{u}{x} = \frac{u}{x} \cdot \frac{v}{v} = \frac{y}{x} \cdot \frac{1}{v} = \frac{C}{v} = \frac{f(v)}{v}.$$

Conversely, if the concentration ratio  $R$  is known, let us say,  $R = \varphi(v)$ , then

$$\frac{y}{x} = \frac{u \cdot v}{x} = v \cdot R = v \cdot \varphi(v).$$

In particular, it follows from Equation 6 that the equation of the concentration ratio is

$$\frac{u}{x} = \frac{A(1 - e^{-kv}) + bv}{v}. \quad \text{Equation 9}$$

Equations 6 and 9 are entirely equivalent and interchangeable. The concentration ratio corresponding to zero diuresis can be determined by calculating the limit reached by the right hand side of Equation 9 as diuresis approaches zero. This limit,  $(Ak + b)$ , is numerically equal to the initial slope of the clearance curve (Equation 8). To the zero clearance there corresponds, therefore, the limiting concentration ratio  $(Ak + b)$ . The correspondence between the concentration

ratio and the clearance is complete at all diureses.<sup>3</sup>

The behavior of the concentration ratio can be followed in Figure 3. The points were obtained from the original data of *Cases 1, 2, 3* and *4*, while the curves are the result of substituting in Equation 9 the already fitted clearances  $C_1$ ,  $C_2$ ,  $C_3$ , and  $C_4$ .

#### LIMITING CONCENTRATION RATIO

Each curve in Figure 3 begins at a definite point, the point we have called the limiting concentration

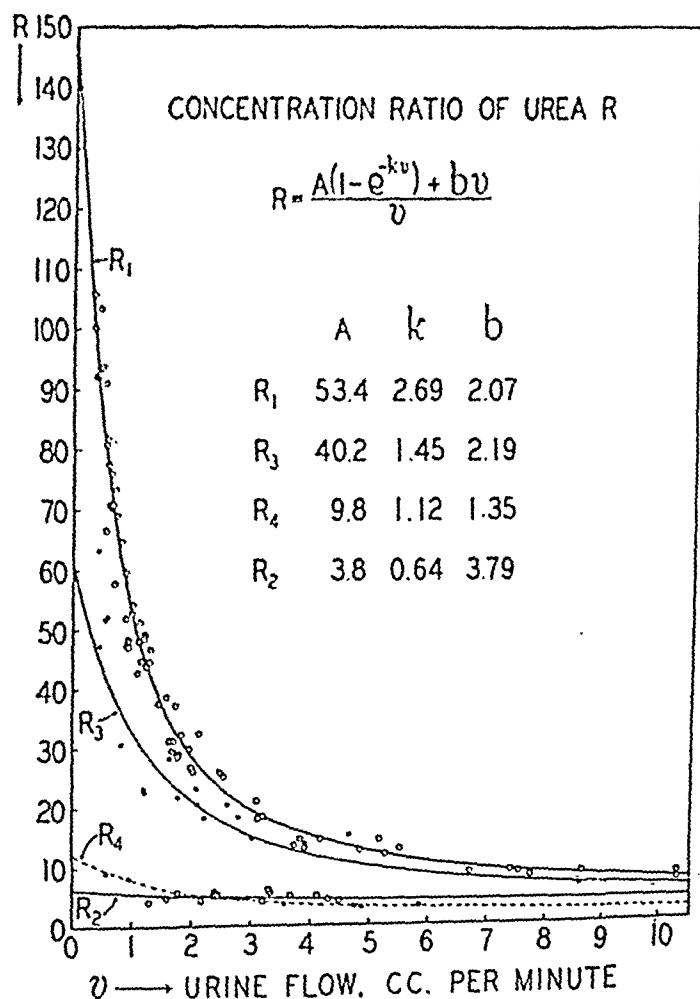


FIG. 3. CONCENTRATION RATIO OF UREA,  $R$ , IN A NORMAL INDIVIDUAL, *Case 1*, AND IN 3 NEPHROSCLEROTICS, *Cases 2, 3* AND *4*.

The cases are identified by the subscripts of  $R$ . The curves begin at the value  $Ak + b$ , the limiting concentration ratio at  $v = 0$ . The large divergence of the curves at  $v = 0$  and their closeness at large diuresis should be noticed. In *Case 2* the concentration ratio,  $R_2$ , is almost constant within the range of diureses observed.

<sup>3</sup> Up to the present time, the correspondence between these two entities has been considered only at urine flows in the neighborhood of 1 cc. per minute, at which flows the concentration ratio is about equal numerically to the clearance (11 to 13).

ratio of urea (7).<sup>4</sup> From this limit on, the concentration ratio lowers quite rapidly in *Case 1*, reaching, at  $v=10$ , a value about 5 per cent of its initial value. In *Case 3*, the concentration ratio begins at 60.5 and at  $v=10$ , has become equal to 6.2, 10.3 per cent of its initial value. In *Case 4*, the concentration ratio is initially 12.5, and at  $v=10$ , has become 2.5, 20 per cent of the former. In *Case 2*, the concentration ratio begins at a very low level, 6.2, and, if allowed to extrapolate to  $v=10$ , it will become equal to 4.2, 67 per cent of its initial level. At  $v=0$ , the concentration ratio of *Case 1* is 2.4 times that of *Case 3*, 11.7 times that of *Case 4*, and 23.4 times that of *Case 2*; at  $v=1$ , the concentration ratio of *Case 1* is, in the same order, 1.6, 6.6, and 9.3 times that of the others; and at  $v=10$ , it is, in the same order, 1.2, 3.0, and 1.8 times that of the others. The really discriminating part of the curves is therefore the part that corresponds to small diuresis, and the smaller the diuresis the better.

This conclusion is quite contrary to the opinion of Chasis and Smith (10), who think it advisable to maintain the urine flow above 1.5 cc. per minute when utilizing the excretion of urea as an index of renal function, "since at flows below that level complicating factors (dehydration, lowered filtration rate, etc.) may vitiate any empirical mathematical correction." It is not clear in the paper of Chasis and Smith what part is played by "dehydration, lowered filtration rate, etc." on the effect which a diuresis less than 1.5 cc. per minute has on the clearance, or what difference, if any, exists between the effect of these factors in renal disease and in health. Yet, in so far as a comparison between the normal and the abnormal

states of renal function is concerned, it would seem immaterial which the effects of the factors mentioned by Chasis and Smith might be. If they have the same effect on both the normal and the abnormal clearances, the effect will pass undetected, and if they have a different effect, why not make use of it to differentiate further the two cases? Besides, the curves shown in Figures 1, 2, and 3 are so smooth that no source of trouble appears critically at 1.5 cc. per minute or at any other diuresis.

While we do not find any good reason for restricting our observations to urine flows greater than 1.5 cc. per minute, a glance at Figure 3 will show that such restriction in diuresis will leave unused one-half or more of the available scale of the concentration ratio.

Consequently, in order to utilize to the fullest extent the information afforded by the great concentrating power of the normal kidney, the urea excretion in renal disease should be compared with the normal at the smallest possible diuresis. For example, the limiting concentration ratio in *Case 1* is 145.7, in *Case 2* is 6.2. In other words, the damage to the kidneys of this nephrosclerotic woman is such that their power to concentrate urea has been reduced to only four-hundredths of that of our normal subject. This statement should be contrasted with the per cent comparison made earlier in the paper between the clearances of *Case 1* and *Case 2*. According to this comparison, the clearance of *Case 2* is, at 1 cc. per minute, 11 per cent of that of *Case 1*, and, at 4 cc. per minute, 30 per cent. In view of the almost total loss of the power of the kidneys of *Case 2* to concentrate urea, the inadequacy of the information given by the clearance at 4 cc. per minute should be apparent.

We feel justified in suggesting the limiting concentration ratio as a most sensitive index of urea excretion.

#### BEARING OF THE CONCENTRATION RATIO ON CONCENTRATION TESTS

The foregoing considerations have their counterpart in the concentration tests of renal function. The low and fixed values of the specific gravity of the urine correspond to the lowering and the small variation of the concentration ratio. Indeed, a

<sup>4</sup> It should be noticed that Equation 2, Table I, does not lead to a definite limit for the concentration ratio (7), while Equation 1, Table I, leads to the constant  $u/x = p$ , that is, a constant concentration ratio at all diureses below 0.35 cc. per minute. This constancy means, graphically, that the curve of the concentration ratio, growing higher and higher as diuresis diminishes, will bend suddenly at  $v=0.35$  cc. per minute, and, at smaller diureses, will become parallel to the axis of diuresis. This unlikely result shows that the limiting concentration ratio arrived at by fitting a straight line to the clearance at small diuresis does not give a close approximation to this limit. A closer value may be found by linear extrapolation from the concentration ratios at low diuresis, as can be seen in Figure 3.

close correlation between these tests has been verified in both moderate and pronounced impairment of renal function (14), but, according to several reports (14, 15), the correlation is not strong when the impairment is slight. Because this question has considerable practical importance, we have started a reinvestigation of it. Even though this investigation is not finished, we are prepared to make a few preliminary remarks.

At present, the majority of the usual concentration tests determine the specific gravity of the urine at the smallest possible diuresis, while the clearance is determined at any diuresis, large or small, but rarely at the smallest possible diuresis. In addition, if diuresis is less than 2 cc. per minute, the usual practice consists in referring the clearances to the value they would have at 1 cc. per minute, by a formula derived from Equation 2, Table I.

Since the specific gravity of the urine depends on diuresis, and since both the clearance and the concentration ratio of urea depend also on diuresis, it does not seem quite correct to compare the specific gravity at a diuresis of, say, 0.2 cc. per minute, with the concentration ratio—or its equivalent, the clearance—at 1 cc. per minute, or at larger diureses. When this situation is looked at from the standpoint of the concentration ratio, it appears almost self-evident that in order to make a satisfactory comparison both tests should be carried out at the same diuresis.

#### POSITIVE SLOPE OF THE CLEARANCE AT LARGE DIURESIS

The examination of the concentration ratio at large urine flow may give us some insight into the significance of the constant  $b$ . Figure 3 shows that, at large urine flow, the curves become close to one another, and it suggests that, if the urine flow can be made sufficiently large, the curves will become indistinguishable. According to the maximum clearance concept, the concentration ratio could become, at some large value of diuresis, not only equal to, but even less than 1. According to the new formulation (Equation 6), the concentration ratio becomes, at large diuresis,

$$\frac{u}{x} = \frac{A + bv}{v},$$

and this expression approaches the value  $b$  as diuresis becomes larger and larger. Since the concentration of urea in the urine should be, at the least, equal to that in the blood, we conclude that  $b$  could not be zero and should not be less than 1. In all the examples given in this paper,  $b$  is larger than 1. In Section 3, we have presented a theoretical argument showing that the urea clearance of a kidney which does not concentrate urea should rise linearly with diuresis, with a slope not less than 1. In the examples of Chasis and Smith (10), cited here in Footnote 2,  $b$  is larger than 1. In the diagrams of Shannon (16), the slope of the linear part of the urea clearance of the dog is also larger than 1.

It may not be without interest to notice that the data of Chasis and Smith (10) on the excretion of inulin in man, and those of Shannon (16) on the excretion of creatinine in the dog, show likewise an upward trend in the clearance of these substances as diuresis increases. This evidence suggests that the inference we have drawn from the behavior of the concentration ratio of urea at large diuresis may be generalized to other substances excreted in the urine.<sup>5</sup>

#### SUMMARY

The urea clearance in man rises continuously with diuresis at all diureses, both in health and in

<sup>5</sup> Shannon (16), and Chasis and Smith (10), although acknowledging the variation of the creatinine and inulin clearances with urine flow, have attempted to explain away this variation, by such considerations as hydration, dehydration, etc. These authors determined neither the slope of the linear part of their clearances nor the standard error of the slope. Had Shannon computed this standard error he would have been enabled to estimate the significance of the slope, and to conclude whether the slope of the creatinine clearance in the dog is significantly different from zero or not. However, Shannon states (16) that the creatinine clearance is essentially constant between 0.5 and 4 cc. per minute, and brings in support of this statement the fact that the largest increase in the clearance, in per cent of the clearance at 0.5 cc. per minute, is very small in comparison with the per cent increase in the urine flow. This method of comparison, which amounts to making the significance of the slope  $b$  in a regression equation of the form  $y = a + bx$  dependent on the value of  $a$ , is incorrect. The significance of the slope  $b$  does not depend on  $a$ . In fact, the formula of the standard error of  $b$  does not contain the term  $a$  (17).

renal disease. This effect of diuresis occurs in nephrosclerosis, both with intact renal innervation and after denervation.

The relation between the clearance and diuresis is adequately represented by the equation

$$C = A(1 - e^{-kv}) + bv,$$

in which  $C$  stands for the clearance,  $v$  for diuresis,  $A$ ,  $k$ , and  $b$  are constants, and  $e$  is the base of the natural logarithms. This equation has been fitted to the data of 1 normal subject and of 3 patients with nephrosclerosis.

The equation contains as a particular case the approximate proportionality of the normal clearance to diuresis when the latter is less than 0.35 cc. per minute. At diureses between 0.35 and 2 cc. per minute, the equation represents the normal clearance better than the commonly used square root relation. At larger diureses, the equation becomes a straight line with slope  $b$ . In the 4 examples given, the constant  $b$  is shown to be significantly different from zero.

The existence of the constant  $b$  and the diminution in the value of the constant  $k$  in renal impairment explain the change in the shape of the clearance curve in renal disease, and render invalid the estimation of the abnormal clearance in per cent of a normal clearance, when the urine flow is not specified.

It is proved that the concentration ratio of urea and the clearance of urea are mutually equivalent at all diureses, and that, if the equation of one of them is known, the equation of the other is known.

The limiting concentration ratio, that is, the limit approached by the ratio of the urea concentration in the urine to the urea concentration in the plasma as the urine flow approaches zero, is computed. This limiting value, equal numerically to the initial slope of the clearance curve, is recommended as a most sensitive index of urea excretion.

By virtue of the correspondence between the clearance and the concentration ratio of urea, it is shown that, when comparing the concentration tests of renal function with the urea clearance, both the specific gravity of the urine and the clearance—or its equivalent, the concentration ratio—should be compared at the same urine flows.

Since the concentration of urea in the urine cannot become less than that of the blood, it is

inferred that the concentration ratio at large diuresis should have the limit 1. Under these conditions, the urea clearance should have at large diuresis a slope greater than zero, and when both the clearance and the urine flow are computed in the same units, the slope should be at least equal to 1. This conclusion is borne out by the data presented in this paper and by the data of other investigators. The suggestion is made that this conclusion also applies to the clearance of other substances, such as inulin and creatinine.

We wish to acknowledge our indebtedness to Professor Harry Goldblatt, School of Medicine, and Professor Charles Rehor, Cleveland College, both of Western Reserve University, for their careful revision of the manuscript.

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# STUDIES ON RENIN: THE DURATION OF THE PRESSOR EFFECT OF LARGE DOSES IN CONSCIOUS NORMAL AND RENALLY ABNORMAL DOGS. OBSERVATIONS ON ANESTHETIZED AND UREMIC DOGS, AND THE ANAPHYLACTIC AND PATHOLOGICAL EFFECTS OF PIG RENIN

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The following investigation was started on the assumption that the injection of renin in adequate amounts might have a greater effect in dogs with experimental renal "ischemia" of the Goldblatt type than in normal dogs, provided that endogenous renin were responsible for the experimental hypertension. Trained, unanesthetized dogs were used in order to eliminate the disturbing effects of anesthesia on vasomotor regulation. Constriction of one or both renal arteries, partial obstruction of one ureter, single nephrectomy, and combinations of these procedures were carried out on a series of dogs to furnish the background of renal abnormality. Homologous and heterologous (pig) kidney<sup>1</sup> extracts were injected repeatedly into normal and renally abnormal dogs, in single or multiple doses, or as continuous injection by means of a pump. For purposes of comparison, renin was also injected into a series of uremic dogs and a group of anesthetized, renally normal dogs. The use of heterologous renin furnished observations on foreign protein reactions and certain pathological changes.

## METHODS

*Preparation of kidney extracts.* After removal from the animal, the kidneys were placed immediately on carbon dioxide snow and packed in the ice overnight. The following morning the frozen kidneys were weighed, allowed to soften, and then ground into a volume of 154 mM. NaCl, equivalent to 4 times the total weight of the kidney tissue. Next, the mixture of ground kidneys and saline was placed in quart jars and shaken on a mechanical shaker for 30 minutes, after which the contents were strained through several thicknesses of gauze. The fil-

trates from the separate extractions were mixed in one large container for the purpose of obtaining a single solution. This solution was placed in 60 cc. centrifuge tubes and heated in a 55° C. constant temperature bath for 20 minutes, the contents being stirred intermittently (1). The tubes were removed immediately and centrifuged, after which the clear filtrate was decanted (Step 1).

Two hundred and fifty cc. lots of the filtrate were placed in "Visking" cellulose sausage casings (size 1½ in.), and fanned to dryness, which required about 12 hours. The dried casings with contents were kept in vacuum desiccators over H<sub>2</sub>SO<sub>4</sub> until ready for use. The dried casings when needed were cut into one inch lengths, placed in a large beaker and water added to dissolve the dried residue. The casing strips were then squeezed out and removed (Step 2). Five-tenths N HCl was added to the dark brown solution to a final pH of 4.2 to 4.3, a heavy precipitate separating. After centrifugation, the filtrate was placed in 50 cc. centrifuge tubes and heated in a 55° C. constant temperature bath for 10 minutes, the contents being stirred every few minutes. After centrifugation, the centrifugate was placed in a casing and dialyzed against water overnight in the ice room, a heavy precipitate separating. The next morning the bag was emptied and the mixture centrifuged (Step 3). An equal volume of saturated ammonium sulfate was added to the clear filtrate. The precipitate was collected by centrifugation. If a further purification is required, the ammonium sulfate precipitation can be repeated. The final precipitate was dissolved in water, placed in a casing, and dialyzed against water overnight. The following morning the casing was opened, the contents centrifuged, and the clear, nearly colorless filtrate was diluted (Step 4). One cubic centimeter of the final solution corresponded to 20 grams of kidney.

Most of the extracts were analyzed for total nitrogen by the micro Kjeldahl method (2).

*Measurement of blood pressure.* The dogs selected for this work were tractable animals which could be trained to lie quietly for an hour or two on a board with their legs tied. The weights of nearly all the dogs fell between 9 and 15 kgm., and their control mean blood pressures, between 110 and 140 mm. Hg. The mean blood pressure was recorded on a kymograph by means of a mercury

<sup>1</sup> We are deeply indebted to Dr. David Klein, Wilson Laboratories, Chicago, for the pig kidneys used in this study and for some of the extracts.

manometer, connected through a large glass cannula with a gauge 18 needle introduced, by direct puncture, into a femoral artery. Sodium citrate solution, 2.5 per cent, was used as the anti-coagulant in the system. Ordinarily, the blood pressure tracing could be run for 10 or 15 minutes with each arterial puncture. In many instances, records of 20 to 30 minutes, at times 40 to 60 minutes, were obtained before a clot obstructed the needle. During the sharp rises in blood pressure resulting from the injection of renin or in the course of the longer tracings, small amounts of citrate solution were momentarily introduced into the cannula, from the pressure bottle, to prevent coagulation. Before the injection of renin, sufficient time was allowed for the blood pressure to be stabilized.

The animals were carefully observed as to changes in heart rate, respiration, nervous behavior, or other symptoms. Blood pressure records from experiments unsatisfactory because of restlessness of the animal, difficult arterial punctures, foreign protein reactions of some severity, or other extraneous vasomotor factors, have been excluded from the results. On the basis of experience, the blood pressure was considered to have returned to the control level when it was within 10 or 15 mm. of the original reading. A residual elevation of 20 to 25 mm. was considered as borderline. Residual elevations of 30

or more mm. were considered as significant hypertension if observed at 60 or more minutes after the end of the injection of renin.

*Operative procedures.* The constriction of renal arteries was carried out according to the Goldblatt technic (3). Constriction of the ureter to produce hydronephrosis was accomplished by means of a Goldblatt clamp or by the technic previously described (4). Nembutal anesthesia was used in all but a few dogs.

## RESULTS

*Pressor responses to single doses of renin.* Figure 1 illustrates the pressor effects of single doses in 24 representative experiments, 11 of which were on normal dogs, and 13 on dogs with renal abnormalities. The amount of renin injected was enough to produce a rise of at least 50 to 60 mm. of mercury in the mean femoral blood pressure. There was little difference in the immediate response of a renally normal and abnormal dog to single moderate or large doses of dog or pig renin if allowance was made for differences

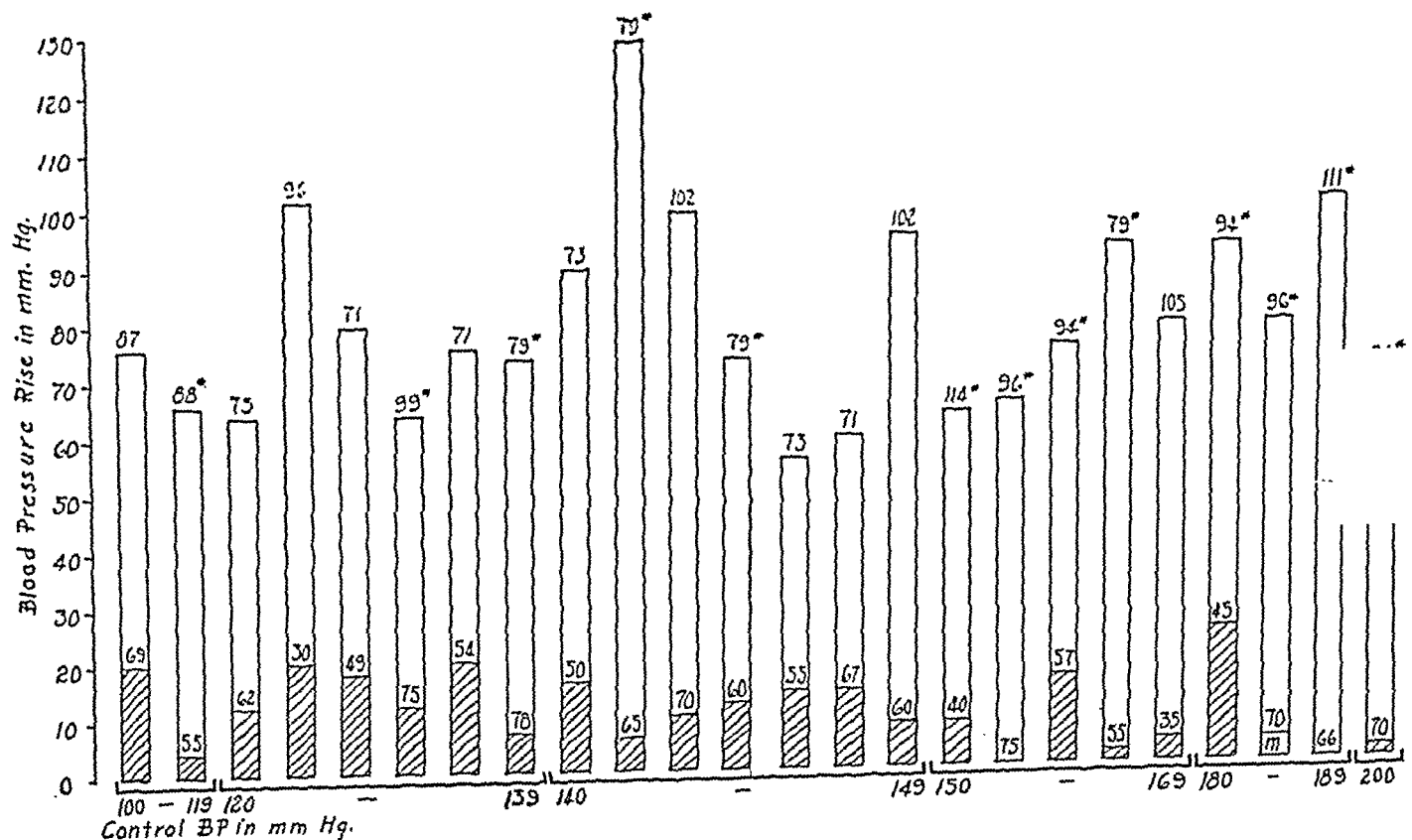


FIG. 1. NORMAL DURATION OF PRESSOR EFFECT OF MODERATE OR LARGE SINGLE DOSES OF RENIN IN NORMAL AND RENALLY ABNORMAL DOGS

Each column represents a single experiment on the dog whose number is listed above it. The asterisk indicates a dog with abnormal kidneys, i.e., constriction of artery or ureter, or nephrectomy. The height of the column is the peak rise in blood pressure, shortly after the injection. The horizontal line in the lower part of each column shows the level of the residual hypertension at the time, in minutes, after the injection denoted by the figure above the bar. The letter "m" indicates a minus value.

TABLE I

The pressor effect of large single doses of renin in normal and renally abnormal dogs

Dog number	Kidney extract		Blood pressure				Renal abnormality	
	Nitrogen per cc.	In- jected	Control	Elevation			Left	Right
				Peak	Terminal			
	mm. per cc.	cc.	mm. Hg	mm. Hg	mm. Hg	minutes	nephrectomy or clamps	
73	9.5	5*	140	90	16	50	None	
102	0.97	4	142	100	10	70	None	
	0.97	3.5	146	96	6	60		
79	0.54	4	140	130	6	65	N†	
85	1.05	20**	104	104	22	60	U	
		4	114	100	14	52		
	0.31	6	146	92	22	60	U	U
111	2.70	9	186	102	0	66	U	A
99	0.97	2.5	102	96	20	80		A
69	1.05	20**	172	90	22	60	A	A; U
94	0.31	6	145	132	42	60	A	
65	9.5	4*	140	140	30	90	N	A; U
	10.5	4*	150	110	40	80		

\* Dog kidney extract carried through 2 stages of preparation.

\*\* Dog kidney extract carried through 4 stages of preparation.

† N = Nephrectomy; U = Ureter; A = Artery.

in the control blood pressure levels in the 2 groups of dogs. The duration of the pressor effect was also similar in the 2 series, the blood pressure returning to the control level in 40 to 60 minutes. It should be noted that, for practical reasons, time recorded for the return of the blood pressure to the control level was not necessarily the minimum figure.

The pressor effect of very large single doses of renin, manifested in peak rises of 90 to 140 mm., is illustrated in Table I. In 6 experiments on 2 normal and 3 renally abnormal dogs, the blood pressure returned to the control level within an hour. Borderline residual elevations of 20 to 26 mm. were recorded in 3 with bilateral constriction of arteries or ureters. Residual hypertension of significant degree, 30 to 42 mm. at 60 to 90 minutes after the injection of renin, occurred in 2 dogs: Dog 68 with a nephrectomy and both artery and ureter constricted in the remaining kidney, and Dog 94 with one artery constricted.

*Pressor responses to multiple doses of renin.* The effect of multiple injections of potent doses of renin varied with the amount of the individual dose, the time interval between injections, and other factors. Tables II and III and Figures 2

and 3 illustrate the salient features. Prolonged duration of renin activity in certain renally abnormal dogs was first noted in response to multiple injections of kidney extract.

In general, the injection of several doses of renin, within a total period of 10 to 20 minutes, produced about the same ultimate effect as a single large dose. When 10 or more minutes elapsed between injections, the intervening hypertensive plateau was better maintained in both normal and renally abnormal dogs, but in all of the former, and in the milder types of the latter, the blood pressure was recorded at or near the control level within 30 to 80 minutes after the last dose of renin.

However, a *prolongation of renin action* was demonstrated in a delayed return of the blood pressure to the control level after the last injection in dogs with repeated bilateral clamping of the renal arteries, constriction of one or both arteries and one ureter, or with constriction of artery or ureter to one kidney and opposite nephrectomy (Table III, Figure 3). The injection of multiple doses of renin in these animals resulted in a hypertensive plateau of 40 to 78 mm. Residual elevations of 28 to 66 mm. were recorded at periods of 57 to 114 minutes after the last dose of renin.

*Pressor responses to continuous injection of renin.* The effects obtained from multiple doses of renin were, on the whole, duplicated in experiments involving the continuous injection of renin (Tables II, III; Figures 4, 5). The renin was, with few exceptions, injected undiluted or in a dilution of 1 to 2. The rate and duration of the injection were varied comparably in normal and abnormal dogs, in so far as practicable. The exclusion of experiments with anaphylactic reactions has limited the data on the various groups of dogs shown in the tables and figures, although the general features of the pressor response were similar.

The continuous injection in 6 normal dogs of 0.2 to 1.2 cc. of renin per minute over a period of 39 to 94 minutes, resulted in the maintenance of a hypertensive plateau averaging from 20 to 34 mm. in 5 dogs, and 52 mm. in 1 dog. The first 20 minutes of the blood pressure record was not included in the "plateau" but considered as the effect of the initial dose of renin. Upon cessation of the injection, the blood pressure returned

TABLE II

*The normal duration of the pressor effect of multiple doses or continuous injection of renin in representative normal and renally abnormal dogs*

Dog number	Kidney extract nitrogen	Kidney extract injected					Blood pressure					Renal abnormality		
		Initial	Multiple		Continuous	Time	Control	Elevation				Left	Right	
			cc.	number				cc.	cc.	mm. Hg	mm. Hg			mm. Hg
73		8*	1	20		18	130	64		12	45	nephrectomy or clamps None		
96	0.31	2	3	6		10	130	70		20	44	None		
99	0.45	2	1	8		15	104	92		24	60	None		
103	0.91	2	5	10		61	168	62	20	-6	45	None		
113	0.26	3	4	9.5		60	122	74	36	14	75	None		
71	0.45	2	1	8		15	168	100		4	50	A†	A	
64	0.91	2	3	6		38	220	60		16	32	A		
102	0.91	2	5	10		56	166	102	35	0	79	A		
106	0.37	2	4	9		42	142	88	38	8	74	U		
73		2				93†	126	86	33	20	53	None		
96	0.91	2				15	142	90	52	4	39	None		
120	2.70	3.5				48	134	68	24	14	71	None		
125		2				44‡	98	90	20	8	0	None		
L 5	1.61	3.7				50§	156	72	25	-10	42	None		
128		2				43	146	72	34	14	83	None		
		3				45	152	58	27	2	19			
90	1.30	2	2	4		127†	160	100	30	20	21	A	N	
94	1.30	2				76†	180	100	40	22	14	A		
	0.75	2				60‡	196	80	30	18	63	A		
94		4				75‡	178	86	45	26	42	A		
104	0.26	4				60‡	168	60	34	16	88	U	A	
105						64	81	228	92	24	45	A	A	
	2.76					53	73	204	78	34	77			
120	0.53	4				67	52	164	136	30	-8	38	A	A
121	1.61	4				54	62	196	68	30	4	45	A	A
124		2				50	83	162	66		-4	0	U	A
111	0.26	4				50‡	48	178	64	20	10	52	U	
	0.53	4				28	41	140	54	36	18	59		
114						76	93	174	68	30	8	77	U	A
115		4				70‡	60	150	100	25	0	5	A	U

\* Dog kidney extract carried through 3 stages of preparation.

† Dilution of 1 volume of kidney extract to 5 volumes.

‡ Dilution of 1 volume of kidney extract to 2 volumes.

§ Dilution of 1 volume of kidney extract to 1.33 volumes.

¶ A = Artery; N = Nephrectomy; U = Ureter.

TABLE III

*The prolonged pressor effect of multiple doses or continuous injection of renin in certain renally abnormal dogs*

Dog number	Kidney extract nitrogen	Kidney extract injected					Blood pressure					Renal abnormality	
		Initial	Multiple		Contin-uous	Time	Control	Elevation				Left	Right
								Peak	Plateau	Terminal			
	mgm. per cc.	cc.	number	cc.	cc.	minutes	mm. Hg	mm. Hg	mm. Hg	mm. Hg	minutes	nephrectomy or clamps	
69	0.79	44*	2	135		220	144	100	50	56	70	A†	A; U
		4**	8	29		220	152	90	78	76	57		
88	0.35	2	3	7.6		48	122	102	54	42	114	U	
103		4*	29	29		79	170	130	50	54	81		
104		3	14	19		45	156	74	45	34	70	U	N
68	0.91	2	1	2	20	107	142	100	70	54	80	N	A; U
103	0.82	3	6	6	74†	150	156	110	60	48	60		
103	2.7				70	140	190	62	44	34	85	A	A; U
	1.68	3			101	117	180	76	40	34	118		
103		3***			43	92	152	80	45	40	182		
90		2			115†	103	156	118	43	54	264	A	N
105	1.61	3			68‡	93	200	136	45	34	125	A	A
	0.87	2.5			88**	94	166	96	50	42	76		
111	0.53	3			124	233	124	64	60	32	97	U	A.
126		3*			47	103	152	64	60	28	57	A	
	0.61	3			75	141	146	96	60	44	75		
127	1.35	3			65	69	150	110	45	48	51	A	

\* Dog kidney extract carried through 1 stage of preparation.

\*\* Dog kidney extract carried through 2 stages of preparation.

\*\*\* Dog kidney extract carried through 4 stages of preparation.

† Dilution of 1 volume of kidney extract to 5 volumes.

‡ Dilution of 1 volume of kidney extract to 2 volumes.

§ Dilution of 1 volume of kidney extract to 1.33 volumes.

¶ A = Artery; N = Nephrectomy; U = Ureter.

to the control level as quickly as after the end of any single moderate dose of renin (Table II; Figure 4).

Similar pressor responses were obtained upon the continuous injection of renin in 11 dogs with various renal abnormalities (Table II). A hypertensive plateau of 30 to 45 mm. was maintained in 10 experiments, of 20 to 25 mm. in 3 experiments. The blood pressure was recorded at or near the control level within the time limits observed in the normal dogs. There was no abnormal duration of renin pressor action in this group of hypertensive dogs.

*Prolongation of renin pressor activity after pump injection* was typically illustrated in 5 dogs with the more severe experimental renal abnor-

malities, but also occurred in 2 dogs shortly after constriction of 1 renal artery (Table III; Figure 5). The renal disturbance consisted of bilateral constriction of the arteries, with additional hydro-nephrosis or unilateral nephrectomy in 4 dogs. A hypertensive plateau averaging 40 to 70 mm. above the control level was maintained during the injection. The blood pressure was still elevated 28 to 54 mm. above the pre-injection figure at 51 to 264 minutes after the end of the injection of renin. The prolonged renin hypertension was reproducible in repeated experiments on the same dog (Table III). Sensitization to pig renin probably played a part in some of the negative experiments on Dog 105.

*Pressor responses to renin in anesthetized dogs.*



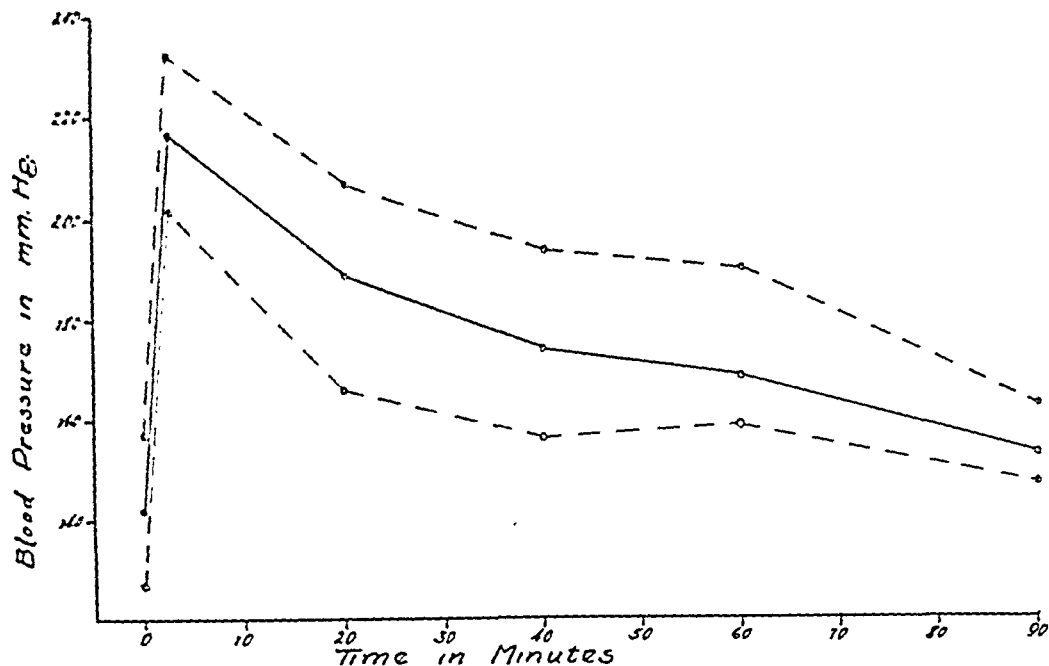


FIG. 4. THE NORMAL DURATION OF THE PRESSOR EFFECT OF CONTINUOUS (PUMP) INJECTION OF RENIN IN NORMAL DOGS

The solid line indicates the average response in 5 experiments. The dotted lines indicate the minimum and maximum range in the series. The injection of renin was maintained for periods varying from 39 minutes to 94 minutes from zero time.

phenomenon regardless of the renal status, the blood pressure of the animal, or the number of experiments on the same dog. The decreasing pressor response on repeated injections of renin varied somewhat with the dosage and the intervals. Occasionally, after several moderate doses of renin, no subsequent pressor response occurred unless the hypertensive plateau had subsided. In the conscious dog, in contrast to the anesthetized dog, successive doses of renin rarely produced step-like rises in the intervening plateaus. However, when renin was injected by pump, changes in the level of the pressor plateau could at times be effected by modifying the rate of injection. When weak, presumably impure extracts were used, tachyphylaxis was apparently accentuated. The failure of a later dose of renin to produce a rise of more than 10 to 20 mm. in the hypertensive plateau did not exclude a prolonged residual effect, as measured by the duration of significant hypertension or the time required for the return of the blood pressure to the control level. The amount of renal extract previously injected was appar-

ently the chief factor in this lag, which rarely exceeded 2 or 3 hours.

*Anaphylaxis to renin.* The use of heterologous pig renin led to anaphylactic reactions in many dogs subjected to repeated experiments. There was only 1 fatality, Dog 105, and in this case there was no immediate shock. The anaphylactic response was best observed on the blood pressure tracing. After the injection of renin in a sensitized dog, the usual rise and peak in the blood pressure occurred. However, the ordinary bradycardia soon gave way to tachycardia, and a more or less steep fall in blood pressure took place within the next few minutes. The level finally reached was above the control value in the mildest reactions, at or near the control blood pressure in the mild reactions, and at varying degrees of hypotension in moderate or severe anaphylaxis. The time of recovery was usually directly related to the depressor response, varying from a few minutes to a half hour or more. After the milder reactions, the experiment could usually be continued and more renin injected without evident



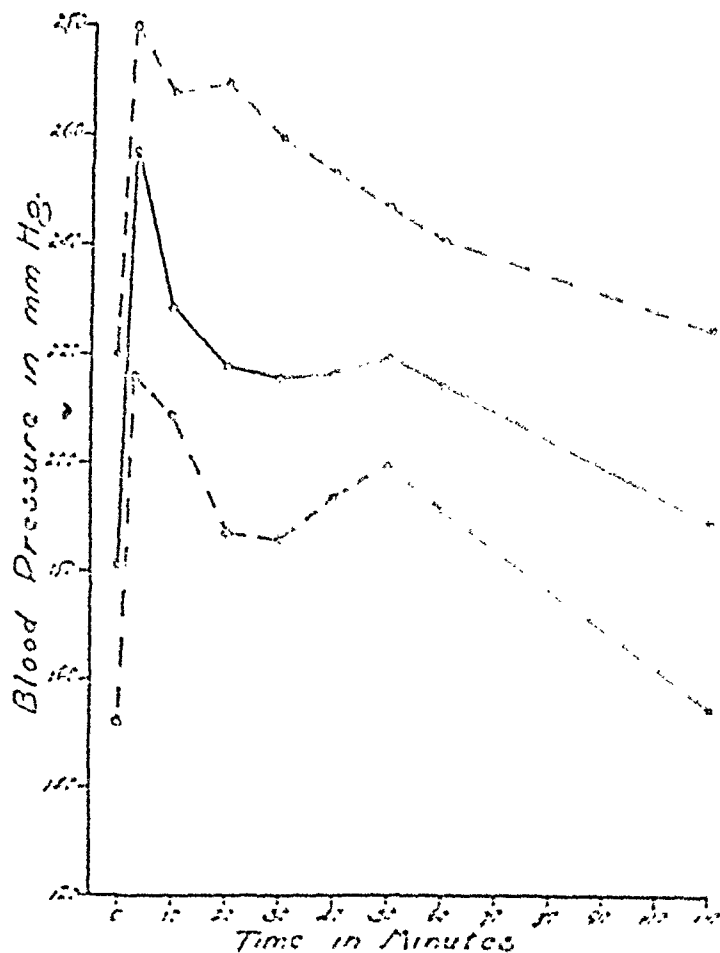


FIG. 2. THE NORMAL PRESSOR EFFECT OF MULTIPLE DOSES OF RENIN IN RENALLY ABNORMAL DOGS

The solid line indicates the average response in 5 experiments. The dotted lines indicate the minimum and maximum range in this series. The interval of time between the first and the last injection ranged from 42 minutes to 80 minutes.

The small series of experiments listed in Table IV is representative of a general reaction pattern. The dosage of renin used was comparable to the amount given to conscious dogs. Even after a single moderate dose of renin, the normal dog under barbiturate anesthesia shows a hypertensive plateau which may persist for a long time. Multiple injections of renin at proper intervals in the anesthetized, renally normal dog can produce successively higher plateaus and prolonged residual hypertension after the last injection. Continuous injection of renin has not been tried.

*Pressor responses to renin in conscious uremic dogs.* As shown in Table V, the injection of homologous renin in single or repeated doses produced a prolonged hypertension in 5 out of 8 non-hypertensive, uremic dogs. Inadequate dosage or improper timing of the injections may have been

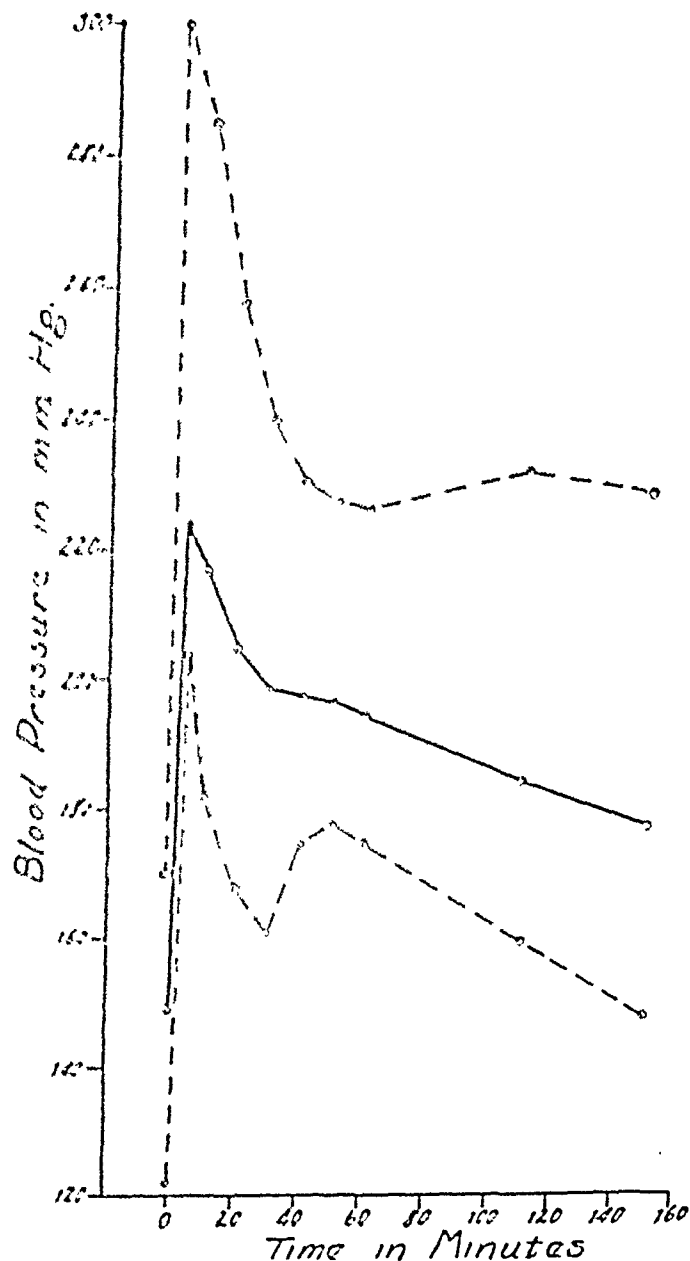


FIG. 3. THE PROLONGED PRESSOR EFFECT OF MULTIPLE DOSES OF RENIN IN RENALLY ABNORMAL DOGS

The solid line indicates the average response in 5 experiments. The dotted lines indicate the minimum and maximum range in the series. The interval of time between the first and the last injection ranged from 45 minutes to 94 minutes.

responsible for the absence of prolonged residual hypertension in the other 3 dogs. The severely hypertensive uremic Dogs 90 and 107 failed to maintain a pressor plateau during or between injections of renin and showed no prolonged residual elevation. Both animals had numerous cardiac hemorrhages and necroses at autopsy. Dog 102, which also had marked hypertension, maintained a pressor plateau of only 20 mm.

*Tachyphylaxis to renin.* This was a constant

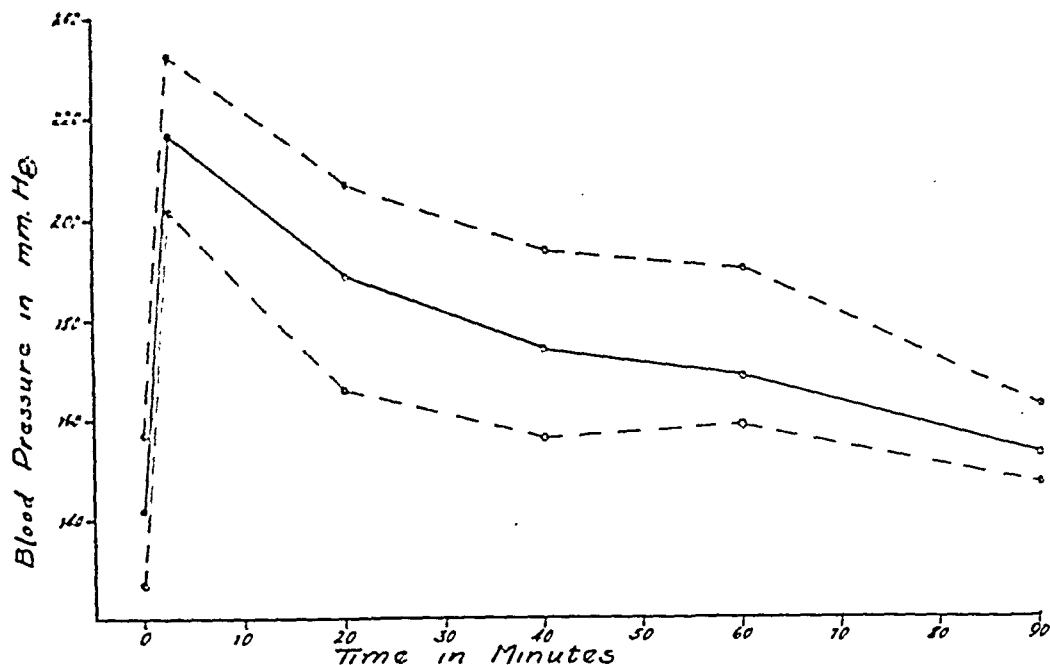


FIG. 4. THE NORMAL DURATION OF THE PRESSOR EFFECT OF CONTINUOUS (PUMP) INJECTION OF RENIN IN NORMAL DOGS

The solid line indicates the average response in 5 experiments. The dotted lines indicate the minimum and maximum range in the series. The injection of renin was maintained for periods varying from 39 minutes to 94 minutes from zero time.

phenomenon regardless of the renal status, the blood pressure of the animal, or the number of experiments on the same dog. The decreasing pressor response on repeated injections of renin varied somewhat with the dosage and the intervals. Occasionally, after several moderate doses of renin, no subsequent pressor response occurred unless the hypertensive plateau had subsided. In the conscious dog, in contrast to the anesthetized dog, successive doses of renin rarely produced step-like rises in the intervening plateaus. However, when renin was injected by pump, changes in the level of the pressor plateau could at times be effected by modifying the rate of injection. When weak, presumably impure extracts were used, tachyphylaxis was apparently accentuated. The failure of a later dose of renin to produce a rise of more than 10 to 20 mm. in the hypertensive plateau did not exclude a prolonged residual effect, as measured by the duration of significant hypertension or the time required for the return of the blood pressure to the control level. The amount of renal extract previously injected was appar-

ently the chief factor in this lag, which rarely exceeded 2 or 3 hours.

*Anaphylaxis to renin.* The use of heterologous pig renin led to anaphylactic reactions in many dogs subjected to repeated experiments. There was only 1 fatality, Dog 105, and in this case there was no immediate shock. The anaphylactic response was best observed on the blood pressure tracing. After the injection of renin in a sensitized dog, the usual rise and peak in the blood pressure occurred. However, the ordinary bradycardia soon gave way to tachycardia, and a more or less steep fall in blood pressure took place within the next few minutes. The level finally reached was above the control value in the mildest reactions, at or near the control blood pressure in the mild reactions, and at varying degrees of hypotension in moderate or severe anaphylaxis. The time of recovery was usually directly related to the depressor response, varying from a few minutes to a half hour or more. After the milder reactions, the experiment could usually be continued and more renin injected without evident

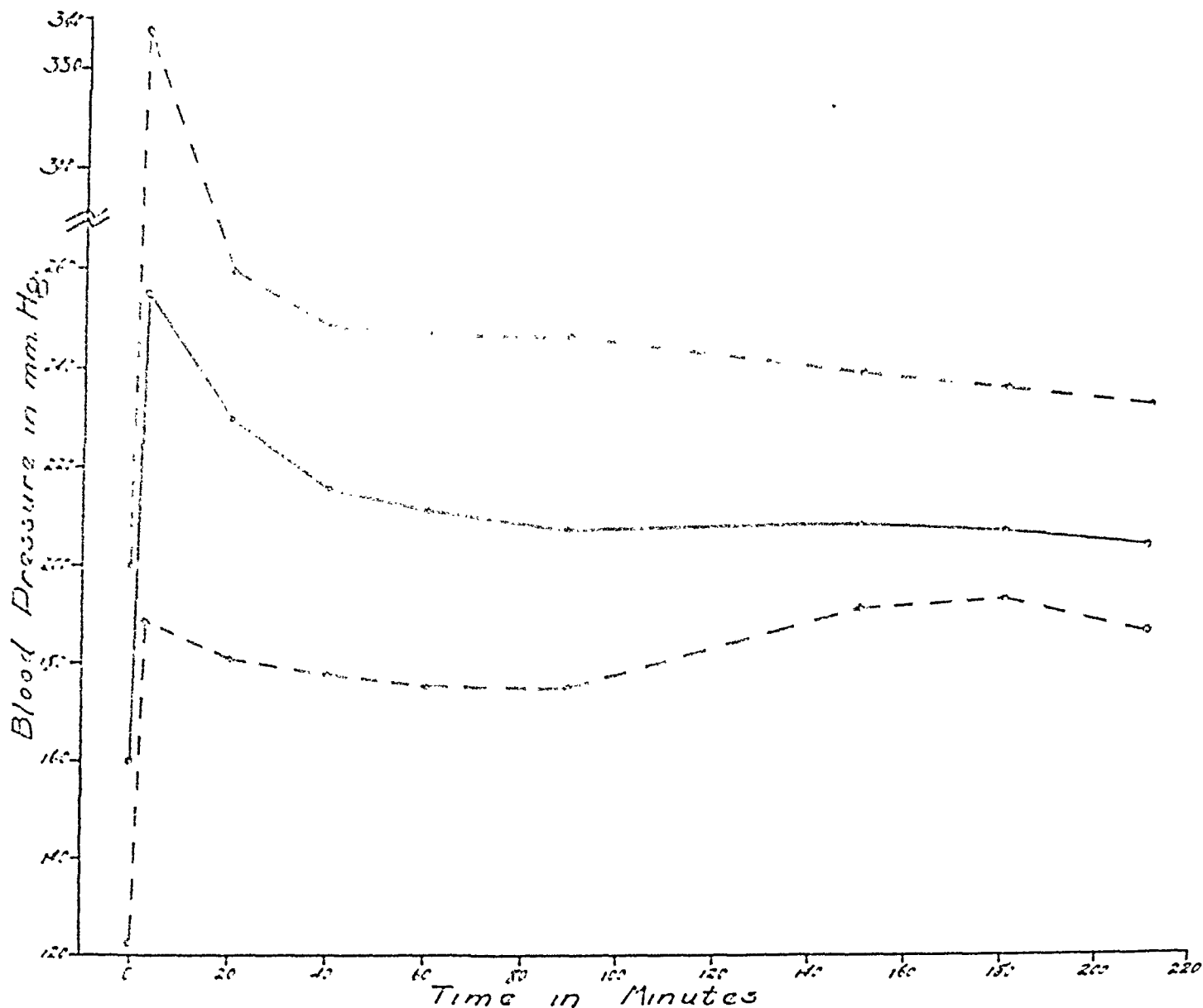


FIG. 5. THE PROLONGED DURATION OF THE PRESSOR EFFECT OF CONTINUOUS (PUMP) INJECTION OF RENIN IN DOGS WITH BILATERAL RENAL ABNORMALITY

The solid line indicates the average response in 11 experiments. Blood pressures on 9 dogs were recorded for 210 minutes, 1 dog for 180 minutes, and 1 dog for 150 minutes. The injection of renin was maintained for periods varying from 69 minutes to 150 minutes from zero time.

abnormal effects. During the more severe anaphylactic reactions, most of the dogs exhibited salivation, and at times vomiting, restlessness, respiratory disturbances, hyperperistalsis, and defecation of normal or diarrheal feces. Tachycardia was a constant sign and, in the more severe reactions, continued for some time after other symptoms had disappeared.

In general, sensitization to kidney extract was determined by dosage and the intervals between injections. Most of the dogs showed some anaphylaxis after a few injections at 5 to 15 day intervals if one large dose was included. In occa-

sional dogs, sensitization was attributable to a large injection of renin, 3 or 4 months previously. The use of kidney extracts weak in pressor activity or high in nitrogen content facilitated the development of the anaphylactic state in dogs previously tolerant of large doses of renin. Desensitization occurred spontaneously in some dogs if a month or two intervened between experiments and if only moderate doses of extract were given. On the other hand, it was possible to desensitize several dogs by producing a mild or moderate anaphylactic reaction a few hours to a day preceding the injection of a large amount of renin. No dog became

TABLE IV  
The prolonged pressor effect of renin in normal anesthetized dogs

Dog number	Kidney extract nitrogen	Kidney extract injected				Blood pressure					Anesthetic
		Initial	Multiple		Time	Control	Peak	Plateau	Terminal		
	mgm. per cc.	cc.	number	cc.	minutes	mm. Hg	mm. Hg	mm. Hg	mm. Hg	minutes	
A	1.05	0.5* 2*	1	1	20	126 116	40 60	40 40	34 40	61 60	Ether Nembutal
B	1.05	1.5*				114	46		28	60	Nembutal
C	1.05	2*				104	48	40	32	107	Nembutal
D		1.6				104	120	65	60	50	Barbital
E		2 0.54	5 3	10 8	123 145	100 74	74† 116†	45 58	54 66	30 33	Nembutal

\* Dog kidney extract.

† Peak occurred with second or later injection.

TABLE V  
The pressor effect of renin in conscious uremic dogs

Dog number	Kidney extract nitrogen	Kidney extract injected					Blood pressure					Renal abnormality		Blood NPN
		Initial	Multiple		Contin-uous	Time	Control	Elevation			Left	Right		
								Peak	Plateau	Terminal				
	mgm. per cc.	cc.	number	cc.	cc.	minutes	mm. Hg	mm. Hg	mm. Hg	mm. Hg	minutes	nephrectomy or clamps		
74	0.99	20†	3	85		107	120	54	24	30	50	N†	U	77
76	10.9	4†	3	10		134	106	84	42	48	64	N	N	266
77	0.85 10.5	20† 2†					120 108	80 62		54 56	50 61	N	N	
80		2†	3	6		71	108	80	30	26	60	N	U	
82	0.90	40†	2	37		41	144	56		18	94	U	N	126
84	0.82	30†	3	90		25	118	62		14	73	U	U	
85	1.24	40†	1	30		17	130	66		16	53	A	A	101
87	1.05	20*					108	132	36	24	96	N	N	79
90	0.58	2			89§	110	200	66	4	8	57	A	N	58
102	0.83	2	5	10		55	204	54	20	10	78	A	A	114
107	0.36	2	4	6		39	200	60		8	21	U	A	80

\* Dog kidney extract carried through 4 stages of preparation.

† Dog kidney extract carried through 1 stage of preparation.

‡ Dog kidney extract carried through 2 stages of preparation.

§ Dilution of 1 volume of kidney extract to 5 volumes.

¶ N = Nephrectomy; U = Ureter; A = Artery.

immune to renin in the sense that it failed to give an immediate pressor response to an intravenous injection of renin. Anaphylactic reactions were never observed in animals receiving homologous renin, whether they were sensitive or not to pig renin.

*Pathological changes following injection of heterologous kidney extract.* In 15 dogs with experimental renal abnormalities, death occurred within a few days after injections of heterologous renin. Two animals, Dogs 79 and 105, died within a few hours after a large continuous infusion of

renal extract, the former showing hemoglobinuria. There were acute degenerative changes in the liver and kidneys. In 12 of the remaining 13 dogs, numerous areas of hemorrhage and necroses were present throughout the heart and gastro-intestinal tract, infrequently in the diaphragm and elsewhere. Histologically, the lesions consisted of hyaline degeneration and fibrinoid necrosis of arterioles and capillaries, with interstitial hemorrhage, leukocytic exudation, and focal necrosis of parenchyma. The kidneys were spared, as was skeletal muscle. While 8 dogs had enough renal insufficiency and hypertension to explain the production of this malignant phase, in 4 dogs with clamps on the renal arteries there was not sufficient renal damage nor severe hypertension to account for the lesions. Thus, Dog 138, with clamps on both renal arteries, had a blood NPN of 25 mgm. and blood pressure of 168 mm. the day before death; Dogs 119 and 120, with blood pressures of 164 and 178 mm., showed no symptoms of uremia but unfortunately had no NPN determination; Dog 121 was killed 5 months after the last injection of pig renin, was in good health except for hypertension, and showed healed lesions in the right auricle. In these animals, the injection of kidney extract apparently was an important factor in the development of the hemorrhagic lesions. Anaphylaxis to kidney extract could not be correlated with the occurrence of the specific pathological changes. No deaths occurred in normal dogs given pig renin or in renally abnormal dogs given dog renin. It is extremely doubtful that they could have survived the extensive cardiac and gastro-intestinal lesions found in the other animals.

#### DISCUSSION

The relationship of renin to experimental renal hypertension has been the subject of much investigation, summarized in recent reviews of the literature (5, 6). The purpose of the present study was to determine whether there was an unusual response to renin on the part of dogs with renal hypertension or other renal abnormality. If renin were responsible for renal hypertension, the injection of extra renin might, under certain conditions, be expected to accentuate the pre-existent hypertension.

The results of this investigation on conscious, trained dogs indicate that renin in single, moderate, or large doses produces essentially similar pressor effects in normal and renally abnormal dogs, regardless of their blood pressure level. However, when multiple doses of renin are given at proper intervals or when continuous injection is employed, the dogs with the more extensive renal abnormalities, such as bilateral constriction of the arteries, or of arteries and a ureter, or with the added load of a nephrectomy, maintain a higher pressor plateau during the administration of renin and show a much longer duration of residual hypertension after the end of the injection. These effects are not observed in normal dogs nor in most dogs with constriction of a single artery or ureter, or with a simple nephrectomy. In these animals, multiple or continuous injection of renin results in a moderate hypertensive plateau, but the blood pressure returns to the control level about as rapidly as after a single dose of kidney extract. The failure of large amounts of renin to produce prolonged hypertension consistently in dogs with extensive renal abnormalities may be attributed to several factors: weak or impure renal extracts; mild or unrecognized foreign protein or anaphylactic reactions to heterologous renin; or a very high control blood pressure close to the limit of sustained vasoconstriction in the individual dog. The effect of extensive cardiac hemorrhages in malignant hypertension in counteracting the pressor response to injected renin must also be taken into account.

One may conclude, therefore, that the unanesthetized dog with extensive renal abnormality and varying degrees of hypertension, but without renal excretory failure, is more susceptible to the action of large amounts of renin than is the normal dog or the dog with unilateral renal abnormality. It is not clear at present whether this behavior indicates an insufficient ability on the part of the normal renal parenchyma to counteract or neutralize the pressor activity of the "ischemic" kidney and the exogenous renin (7, 8), a decreased "anti-pressor" content of the animal's blood (9, 10), or an increased supply of substrate on which renin must act to produce the effective vasoconstrictor (11, 12). Some preliminary observations on the production of "hypertensin" by the action of renin

upon the plasma of normal and renally abnormal dogs, have not revealed significant differences in the amount of plasma substrate in the various animals (13), although they have confirmed its disappearance or marked diminution shortly after the injection of renin (11).

Certain objections to the renin theory of experimental renal hypertension have been raised (14). They concern, chiefly, the development of tachyphylaxis, the similar pressor response to renin of hypertensive and normal animals, the difficulty of maintaining sustained rises in blood pressure on infusion of renin, and the failure of the blood pressure to fall below the control level in either normal or hypertensive rabbits after tachyphylaxis to renin is established. The last objection is a serious obstacle to the renin theory, if tachyphylaxis is considered solely from the point of view of exhaustion of the plasma substrate necessary for renin activity. However, it has been shown that tachyphylaxis is not merely a problem of renin and substrate (15). Furthermore, little is known of the rate of replenishment of substrate or the amount necessary to permit a continuing renin effect *in vivo*. Residual renin hypertension can be present in the absence of demonstrable substrate (13). This may simply mean that the *in vivo* pressor response is a more sensitive criterion than other assay procedures. If, in the intact renal hypertensive animal, the central nervous regulation of the blood pressure is somehow adjusted to the new level (16), this may explain the exact return of the blood pressure to control value after renin tachyphylaxis, since a relatively short period is required to restore substrate for endogenous renin.

If an anti-pressor substance, renin- or angiotonin-inhibitor can be released by normal renal parenchyma (9), the higher pressor plateaus and prolonged residual hypertension produced by injection of renin in renally abnormal dogs may be explained as an impairment of this function. Since prolonged duration of renin activity can occur in renally "ischemic" dogs with only moderate or little hypertension, one must assume a balance between low endogenous renin and inhibitor in these animals, with an inability to produce the latter rapidly in response to a sudden excess of exogenous renin. On this basis, the occurrence of

tachyphylaxis may be taken as evidence against the view that a renin- or angiotonin-inhibitor mechanism is largely responsible for this phenomenon.

Whatever the means by which renin pressor activity is kept within moderate limits after the initial rise in the renally normal, conscious dog, a marked impairment of this function occurs in the anesthetized dog, which behaves as though it had extensive renal abnormality. The prolonged pressor plateau after single injections of renin makes it difficult to evaluate the effects of continuous infusion.

The occurrence of anaphylactic reactions in animals receiving repeated injections of heterologous renin has also been observed in the rabbit (17). The sensitization is apparently not carried over to homologous renin. In this respect, the phenomenon differs sharply from the immunological formation of "anti-renin" in hypertensive dogs, injected intramuscularly with heterologous renin (18). Our anaphylactic hypertensive dogs showed no consistent blood pressure reduction that could be interpreted as evidence of immunity to endogenous renin; but the experimental conditions were not such as to favor the development of "anti-renin."

Foreign protein reactions, as distinguished from true anaphylaxis, were observed in the course of continuous infusion of renin in some dogs. They appear chiefly in the blood pressure response in the case of homologous extracts, resulting in a falling curve that may reach the control level at the end of the injection. Similar reactions have been described in rabbits (19). When heterologous kidney extract causes this type of reaction, severe symptoms and, very rarely, death may occur. It is, therefore, difficult to draw conclusions from experiments with poor pressor plateaus. Some of the discrepancies in the literature on renin activity are undoubtedly attributable to neglect of this factor.

The relation of kidney extracts to the pathology of experimental malignant hypertension has been strikingly emphasized in recent studies on nephrectomized dogs (20). The necessary combination of severe renal insufficiency and hypertension in the etiology of the malignant syndrome of renally ischemic animals (21, 22), has been questioned as

the result of experiments on rats, in which only one renal artery was constricted (23, 24). In the present study, there are definite indications that heterologous renin can precipitate cardiac and gastro-intestinal hemorrhages and necroses, in some dogs without severe hypertension or renal insufficiency. The findings have been detailed elsewhere (25).

The role of renin in human hypertension is still a matter of conjecture. It is plausible to assume renin participation in the severe malignant phase, in which renal ischemia and necrosis provide a source for the pressor substance. Release of renin probably occurs after the sudden occlusion of renal arteries (26). The successful treatment of juvenile hypertension by nephrectomy, in cases of unilateral renal disease or vascular lesions of various types (27), strongly suggests interruption of a renin hypertension. However, direct demonstration of a renin mechanism in this small clinical group is not yet available. If the situation in man is at all comparable to experimental hypertension in the rat (24), renin hypertension of unilateral renal origin would sooner or later lead to a vicious circle because of the damaging effect of hypertension, or renin, on the arterioles of the sound kidney. In ordinary "essential" hypertension, without signs of organic renal involvement, there is, at present, no satisfactory evidence for a renin mechanism. Further study of the pressor response of such individuals to adequate dosage of renin should yield interesting data for comparison with the results observed in normotensive human subjects (28).

#### SUMMARY AND CONCLUSIONS

1. Trained, conscious dogs, injected with renin intravenously, in single moderate or large doses, sufficient to elevate the mean femoral blood pressure 50 to 100 mm. Hg., gave similar pressor responses whether their kidneys were normal or experimentally abnormal as the result of partial constriction of arteries or ureters, with or without unilateral nephrectomy.

2. Multiple doses of renin, or continuous injection by pump, produced a pressor plateau in renally normal and abnormal dogs, but the blood pressure returned to the control level within an hour after the injection in all of the normal ani-

mals and in most of the dogs with unilateral or less extensive bilateral renal lesions.

3. Marked prolongation of renin pressor activity was observed chiefly in dogs with bilateral, experimental, renal abnormalities following multiple doses or continuous injection of kidney extract, free from depressor material.

4. Prolonged duration of renin pressor activity also occurred in anesthetized, renally normal dogs and in the majority of conscious dogs with uncomplicated uremia. Dogs with experimental hypertension and uremia reacted irregularly.

5. Repeated experiments on the same animal with heterologous renin led to the development of anaphylaxis. Dogs sensitized to pig renin reacted normally to dog renin.

6. The pathological lesions of experimental malignant hypertension can be induced or accelerated by the injection of foreign renin, not only in dogs with excessive renal ischemia or necrosis, but in some animals without severe hypertension or uremia.

7. The prolonged effect of renin in conscious dogs with extensive renal abnormality is regarded as evidence in favor of the renin etiology of experimental renal hypertension, and in support of the view that the ratio of normal to abnormal ("ischemic") renal parenchyma is a determining factor in the dog's response to exogenous, as well as endogenous, renin.

8. The relation of renin to human "essential" hypertension remains an open question.

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# THE RELATION OF POSTURAL HEMODILUTION TO PAROXYSMAL DYSPNEA

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## INTRODUCTION

For many years, there has been considerable speculation about the mechanism of paroxysmal dyspnea. Changes in vasomotor, neural, cardiac, and respiratory physiology, nightmares and other trigger mechanisms, and alterations in blood volume have been variously suggested as responsible for the characteristic nocturnal attacks. The present study was undertaken to see whether shifts of body fluid to the blood stream, following rest in the horizontal position, could be correlated with the time of onset of paroxysmal dyspnea and might therefore be considered a factor in inducing acute left-sided heart failure.

Böhme (1) early noted a fall in serum proteins after rest, a rise after periods of exercise; and other workers (2 to 5) have published results indicating that the change from recumbency to an upright position may be followed by a rise in protein concentration, usually ranging from 10 to 20 per cent. Similar changes in the composition and volume of the blood have also been reported (3, 5, 6). Since the introduction of the concept of "cardiac asthma" (7), reviewers of the subject (8, 9) have pointed out the frequently nocturnal character of these episodes, which usually occur at rest and in the horizontal position. Weiss and Robb (9) have suggested that patients with low plasma proteins are more susceptible to severe attacks.

## EXPERIMENTAL AND RESULTS

Serum protein determinations were first made at 2-hourly intervals, throughout the day and night, on a group of 10 normal ambulatory young adults, 10 constantly-recumbent bed patients without heart disease, and 10 ambulatory patients who had chronic cardiac insufficiency, including dependent edema. The serum proteins were determined by a specific gravity gradient tube, Lowry's

modification of the micro-method of Linderström-Lang (10, 11), checked frequently with the micro-Kjeldahl method, and found to measure specific gravity with no greater than a 0.0002 variation. The results are shown in Table I, with the average figures graphically recorded (Figure 1).

It was obvious that a significant and consistent fall in serum proteins, averaging about 0.8 gram per 100 cc., occurred at night in ambulatory individuals, that the values did not rise during the day in those patients confined to bed. Patients with edema demonstrated no greater hemodilution in the recumbent position than did the normals, but it should be remembered that they were of necessity less active. A further confirmation of these changes was obtained by comparing the diurnal serum protein fluctuations of a bed patient with those found a week later when the same patient became ambulatory (Figure 2). Repeated observations on persons staying up all night and sleeping during the day, made it evident that bed rest was associated with an invariable and significant drop in serum proteins from the levels achieved when up and about. Fractionation of the serum proteins showed that the changes observed involved both albumin and globulin equally, although in a larger series significant differences might appear.

In order to evaluate the relative roles of physical activity and position, 5 normal adults rested for 45 minutes in the horizontal position, then walked rapidly for 5 minutes. Blood specimens were taken at the start and close of the exercise period. The same procedure was carried out after a preliminary rest period using a tilt-table, with the patients then raised to 60° for 5 minutes and lying quietly throughout the experiment. Walking caused a rise in serum protein of 0.3 to 0.6 (average 0.48) gram per 100 cc., while a 5-minute change in position without activity produced a rise of from 0.2 to 0.3 (average 0.26) gram per 100 cc.

<sup>1</sup>Dazian Fellow.

TABLE I  
Diurnal serum protein fluctuations

	Case	10 a.m.	12 n.	2 p.m.	4 p.m.	6 p.m.	8 p.m.	10 p.m.	12 m.	2 a.m.	4 a.m.	6 a.m.	8 a.m.
Normals	1	6.5	6.4	6.3	6.6	6.4	7.0	6.5	6.1	5.8	5.7	5.9	6.7
	2	6.3	6.2	6.3	6.3	6.2	6.2	6.3	6.0	5.7	5.5	5.4	5.9
	3	6.8	6.8	6.5	6.6	6.8	6.4	6.1	6.1	5.9	6.0	6.0	6.8
	4	6.7	6.6		6.8	6.8	6.7	6.6	6.3	6.2	6.0	5.9	7.2
	5	7.1	7.1	6.8	7.2	6.9	7.0	7.0	6.8	6.4	6.2	6.2	6.9
	6		6.7	6.7		7.2	6.6	6.5	6.2	6.0	6.0	6.2	7.0
	7	7.1	7.0		7.2	7.3	7.3	7.1	6.9	6.2	6.2	6.3	7.1
	8	7.3	7.2	7.0	7.0	7.2	6.8	6.8	7.0	6.3	6.3	6.1	7.2
	9	6.7	6.7	6.8	6.6	6.8	6.5	6.5	6.5	5.9	6.1	5.9	6.5
	10	6.7	6.8	6.6	7.1	7.1	7.2	6.8	6.9	5.9	5.9	5.8	6.5
Ambulatory cardiacs	1	7.1	7.2	7.2	7.3	7.1	7.1	6.6	6.4	6.3	6.4	6.9	7.1
	2	6.8	7.0	7.2	6.9	6.8	7.0	6.8	6.2	6.0	5.9	6.1	7.0
	3	6.5	6.5	6.4	6.4	6.6	6.5	6.2	5.4	5.5	5.4	5.7	6.3
	4	6.8	6.5	6.6	6.7	6.8	6.6	6.5	6.0	5.9	6.0	5.9	6.7
	5	7.4	7.5	7.3	7.2	7.4	7.5	7.0	6.7	6.6	6.8	7.0	7.5
	6	6.2	6.4	6.5	6.1	6.3	6.3	6.0	5.8	5.7	5.7	5.9	6.3
	7	5.9	5.7	6.0	5.8	5.8	5.8	5.4	5.0	4.9	5.0	5.1	5.8
	8	6.9	7.0	6.9	7.0	6.8	6.8	6.3	6.0	5.8	6.0	6.0	6.8
	9	6.9	6.5	6.7	6.7	6.8	6.6	6.3	6.4	6.2	6.2	6.4	6.7
	10	7.0	7.0	6.8	6.9	7.1	6.7	6.6	6.3	6.0	6.0	6.2	6.9
Bed patients (no heart disease)	1	6.2	6.3	6.4	6.6	6.5	6.5	6.1	6.0	5.7	5.8	5.9	6.6
	2	6.4		6.3	6.3	6.3	6.4	6.4	6.3	6.3	6.2		6.4
	3	6.3	6.2	6.2	6.3	6.3	6.3	6.0	6.1	6.2	6.2	6.3	6.5
	4	6.1	6.3	6.3	5.6	6.0	6.0	6.0	5.9		6.0	5.8	6.0
	5		5.4	5.2	5.3	5.3	5.3	5.4	5.4	5.4		5.5	
	6		4.8	5.3	4.8	4.8	5.0	5.0	5.0	4.6	4.7	4.9	
	7	5.3	5.6	5.6	5.4	5.4	5.2	5.2	5.4		5.4	5.3	5.3
	8	5.4	5.2	5.3	5.3	5.3	5.4	5.4	5.3	5.5	5.3	5.2	5.5
	9	6.1	5.9	6.2	6.3	6.1	6.1	6.2	5.9	5.9	6.1	6.3	6.2
	10	6.5	6.2	6.5	6.4	6.4	6.3	6.5	6.1	6.1	6.2	6.1	6.4

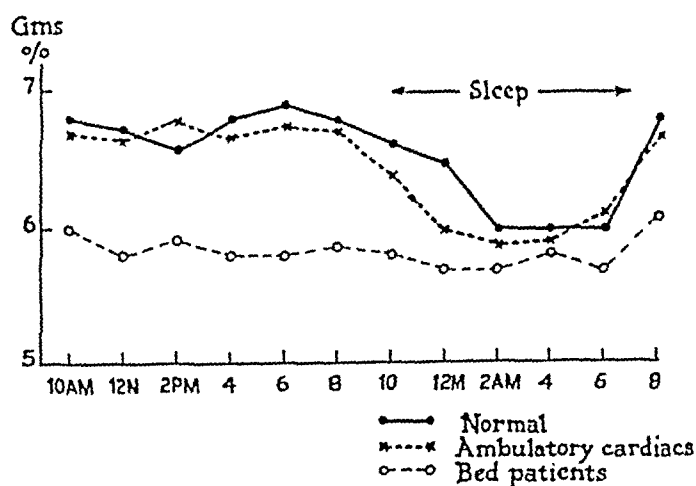


FIG. 1. AVERAGE DIURNAL SERUM PROTEIN FLUCTUATIONS

Simultaneous serum protein, red cell count, hematocrit, mean red cell diameter, vital capacity, and venous pressure determinations were carried out on 10 normal individuals at 4 p.m. and 11 p.m., using venous blood in all instances, the night data being secured after at least one hour of bed rest or sleep. Venous pressures were taken in the

horizontal position, vital capacities while sitting, after the other procedures had been completed. A fall in erythrocyte count of between 180,000 and 370,000, a drop in hematocrit of from 2 to 3 per cent cells in every case, as well as the expected serum protein fall, offered further evidence of hemodilution after horizontal rest. No change in mean corpuscular volume or red cell diameter was observed. In 8 of these 10 normal subjects, there was a 100 to 400 cc. reduction in vital capacity and a 5 to 20 mm. rise in venous pressure after the

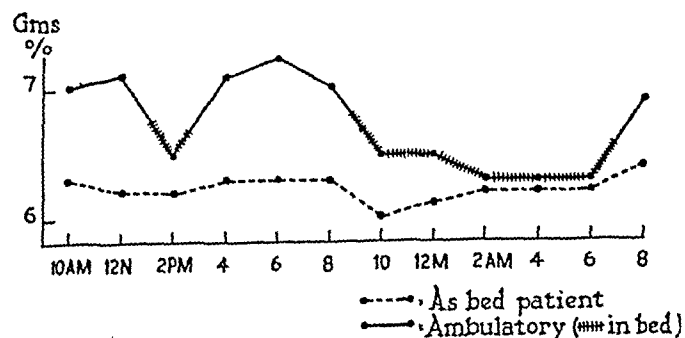


FIG. 2. EFFECT OF ALTERED ACTIVITY ON SERUM PROTEIN VALUES

recumbent position had been assumed for an hour or more.

Finally, serum protein, vital capacity, and venous pressure measurements were made on 8 patients with paroxysmal dyspnea as the presenting symptom of established arteriosclerotic coronary or hypertensive cardiovascular disease. These patients were allowed to be ambulatory during the day, data being secured at 4 p.m., several hours after going to bed (11 p.m. to midnight), and again 10 to 15 minutes after the onset of a characteristic attack (while still sitting up in bed and short of breath).

TABLE II

*Observations on patients with paroxysmal dyspnea*

Case	4 p.m.			11 p.m. to midnight			10 to 15 minutes after onset of paroxysmal dyspnea			Time of attack
	Protein	Venous pressure	Vital capacity	Protein	Venous pressure	Vital capacity	Protein	Venous pressure	Vital capacity	
	grams per cent	mm. H <sub>2</sub> O	cc.	grams per cent	mm. H <sub>2</sub> O	cc.	grams per cent	mm. H <sub>2</sub> O	cc.	
1	7.7	95	2550	6.8	90	2100	7.5	120	1650	2:30 a.m.
2	6.3	80	2800	5.6	100	2500	6.4	150	2300	4:00 a.m.
3	6.5	65	3100	5.5	80	3100	6.4	110	2700	12:50 a.m.
4	6.0	110	2900	5.5	115	2650	6.1	120	2500	3 a.m.
5	6.3	100	2000	5.6	120	1900	6.2	145	1600	3 a.m.
6	5.9	70	2200	5.1	100	2000	5.6	110	1750	4:45 a.m.
7	6.6	100	3400	5.8	110	3050	6.3	130	2800	5:00 a.m.
8	6.3	120	1900	5.6	115	1600	6.0	140	1500	3:15 a.m.

6. From these figures (Table II), it could be appreciated that the usual serum protein drop after bed rest was followed by a significant and rapid rise during the episode of paroxysmal dyspnea, that period in which the patient awakens, and sits or stands up, gasping for air. In other words, not only did the attacks occur at a time when serum proteins were normally found to be reduced, but the maneuvers of the breathless patient, which characteristically alleviate an attack, were those experimentally shown to cause an elevation in serum proteins. Again, in the majority of instances, the vital capacity was found to be reduced after going to bed, while venous pressures tended to increase. These changes became more pronounced during the attacks of dyspnea. Arte-

rial pressures were followed in 3 of these patients. The day and night values were essentially unchanged, 2 of the 3 exhibiting a 15 mm. systolic rise during the paroxysm.

#### DISCUSSION

Many investigators have long been aware of the change in serum protein concentration due to alterations in position and muscular activity, a fact which has not however been emphasized in the clinic. A diurnal fluctuation of 10 to 15 per cent, dependent upon postural factors, has been shown in this study to occur regularly in individuals carrying on a normal existence. This fact offers an explanation for the common observation that the average serum protein values of hospitalized patients are lower than those found in healthy ambulatory subjects. Thus the normal serum proteins, as measured in the recumbent position, are often as low as 5.5 to 6.0 grams per 100 cc., levels frequently regarded as pathological. These changes are often attributed to illness, under-nutrition, shock, or operative protein loss, whereas the position and inactivity of the bed patient may provide an adequate explanation. It should be noted that a significant change can occur in a few minutes, although a longer period is required for a maximal effect.

In view of the comparable alterations in red blood count and hematocrit measurements, which also indicate hemoconcentration in the standing position or during activity, there can be little doubt that the rise in serum proteins results primarily from the loss of fluid from the circulation. Furthermore, plasma volume studies by Thompson and his co-workers (3), showing an 11 per cent drop after standing, and the observations of Gregersen (12), indicating even greater changes after exercise, using the blue dye T.1824, support this idea.

The suggestion of a slightly greater degree of venous and pulmonary congestion in the quiet recumbent state, as judged by venous pressure and vital capacity, is in accord with the concept of a larger and more dilute blood volume at that time, and the possibility that fluids from dependent portions of the body have become redistributed to other sites with a change in position. However, with vasomotor and peripheral factors entering

into the accuracy of venous pressure measurements and with variations in cooperation on the part of the patient in the case of vital capacity determinations, speculation on the lack of parallelism between the serum protein changes and the venous pressure and vital capacity readings appears inadvisable and interpretation of the results must be guarded.

It is generally recognized that paroxysmal dyspnea usually occurs at night or during rest, particularly during the early morning hours, in other words at those times when, in accordance with our observations, serum proteins reach their lowest levels. The effect of the horizontal position upon the resting individual is comparable to the administration of a slow but sustained infusion. It seems probable, therefore, that in a patient already subjected to left ventricular strain by virtue of underlying organic disease, the increase in circulating plasma volume accompanying rest in the horizontal position may prove a significant factor in inducing paroxysmal dyspnea and pulmonary edema. In support of this idea is the evidence presented first by Caughey (13) that infusions or transfusions consistently cause a reduction in vital capacity, and may provoke pulmonary edema in cardiac patients. Murphy and his associates have also shown that an infusion of as little as 100 cc. of a 50 per cent dextrose solution intravenously may have a similar deleterious effect on such patients (14). Furthermore, Burwell (15) has recently shown that a 50 per cent increase in blood volume can cause many of the phenomena of congestive failure in normal dogs.

#### SUMMARY

1. It has been confirmed that serum protein concentrations are considerably altered in health and disease by changes in position and by muscular activity.

2. This decrease in serum protein concentration appears to be the result of hemodilution, due to an increase in plasma volume.

3. The close correlation between nocturnal hemodilution and attacks of paroxysmal dyspnea suggests that an increase in plasma volume is an important factor in the production of acute left-sided failure in individuals with organic heart disease.

4. Clinical interpretations of protein values must be made with caution since an average fall of 0.8 gram per 100 cc. is encountered after rest in the horizontal position.

We are indebted to Doctor Robert F. Loeb for his valuable help in the preparation of this paper.

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# DISTRIBUTION OF SULFONAMIDE COMPOUNDS BETWEEN CELLS AND SERUM OF HUMAN BLOOD

By MARTIN HEINEMANN<sup>1</sup>

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(Received for publication June 10, 1942)

The distribution of sulfonamide compounds between cells and serum of human blood was studied *in vitro*. The rate of transfer from serum to cells, and the effects of temperature and of atmosphere, were investigated. The distribution of these compounds between substrate and ultrafiltrate of serum was also observed.

## METHODS

Freshly drawn blood from healthy human subjects was defibrinated by stirring with a glass rod. If hemolysis was avoided, concentrations in defibrinated and in oxalated whole blood were identical, as were concentrations in serum and in plasma. Cell concentrations were calculated from concentrations in whole blood and in plasma, and from measurements of cell volumes (1). Concentrations were expressed as mgm. per 100 cc. of water in cells and in serum, accepting, for the water of these two media, average figures (1) of 72.3 per cent and 93.5 per cent, respectively.

Since sulfonamide compounds do not dissolve rapidly, their dissolution in serum must be ascertained before the latter is added to cells. By choice of suitably low concentrations, and by incubation at 37° C., this difficulty was overcome.

Sulfanilamide, sulfathiazole, sulfadiazene, and sulfapyridine were measured by the method of Bratton and Marshall (2), using a photoelectric colorimeter.<sup>2</sup>

Substrates and ultrafiltrates of serum were obtained according to Laviets' method (3). It had been established previously that contact with mercury did not affect the determination of sulfonamide compounds in serum.

The effects of erythrocytes and of leukocytes (4) and of an atmosphere of nitrogen (5) upon the ratio of distribution were studied according to procedures communicated previously.

## EXPERIMENTAL RESULTS

The data of Table I represent groups of similar experiments, and demonstrate the effects of time, temperature, and atmosphere upon the ratio of distribution.

<sup>1</sup> Aided by a grant from the John and Mary R. Markle Foundation.

<sup>2</sup> With the Evelyn colorimeter used in this laboratory, filter 540 was employed.

In similar studies, the transfer of ascorbic acid proved to be unidirectional, viz., from serum to cells only. Transfers of sulfonamide compounds, on the other hand, are freely reversible. If from whole blood containing sulfanilamide at a concentration in cells of 13.8, and in serum of 6.9 mgm. per cent, serum was removed and replaced by serum without the drug, an immediate redistribution occurred, the concentrations in cells falling to 8.3 and in serum to 3.0 mgm. per cent. No further change in concentrations was noted after shaking cells and serum at 37° C. for 45 minutes.

Table II shows that sulfonamide compounds attain concentrations of the same magnitude in erythrocytes and in leukocytes.

The ratios of distribution of the four drugs studied are presented in Figure 1, where concentrations in cells and in serum, as stated before, are expressed as mgm. per 100 cc. of water. The

TABLE I  
Effects of time, temperature, and atmosphere upon ratios of distribution

Time after addition	Drug	Temperature	Atmosphere	Concentration in 100 cc. of water	
				Cells	Serum
<i>minutes</i>		°C.		<i>mgm.</i>	<i>per cent</i>
Immediately 20 40	Sulfathiazole	37	Air	2.9	16.5
				2.9	16.5
				2.9	16.5
Immediately 20 40	Sulfanilamide	37	Air	16.5	13.5
				16.5	13.2
				16.3	13.4
30	Sulfanilamide	37	Air	14.9	7.0
30		37	Nitrogen	15.0	7.3
45	Sulfadiazene	37	Air	2.8	9.0
45		9	Air	3.0	9.2

TABLE II

*Independence of ratios of distribution from presence and absence of leukocytes*

Drug	Erythrocytes	Leukocytes	Concentrations in 100 cc. of water	
			Cells	Serum
Sulfanilamide	millions	thousands	mgm. per cent	
	4.2	None	15.7	8.0
	4.0	8.4	15.9	8.5
Sulfathiazol	4.5	None	4.2	12.7
	3.8	10.3	3.8	12.2
Sulfapyridine	4.5	None	7.2	9.1
	3.8	10.3	7.1	9.4

columns on the right hand side show the percentage of each drug ultrafiltrable from serum.

## DISCUSSION

From the data presented in Table I, it is obvious that no gradient of transfer could be detected; analyses of samples taken immediately, and at intervals after serum containing the drug was added to cells, invariably yielded identical concentrations. The reaction associated with the transfer of sulfonamide compounds, therefore, seems to be instantaneous. This holds at 37° C., as well as at icebox temperature. The transfer of sulfonamide compounds from serum to cells could be reversed if the concentrations in the former were reduced experimentally. The presence of oxygen was not required for this reaction, which took place in an atmosphere of nitrogen in exactly the same manner as in an atmosphere of air.

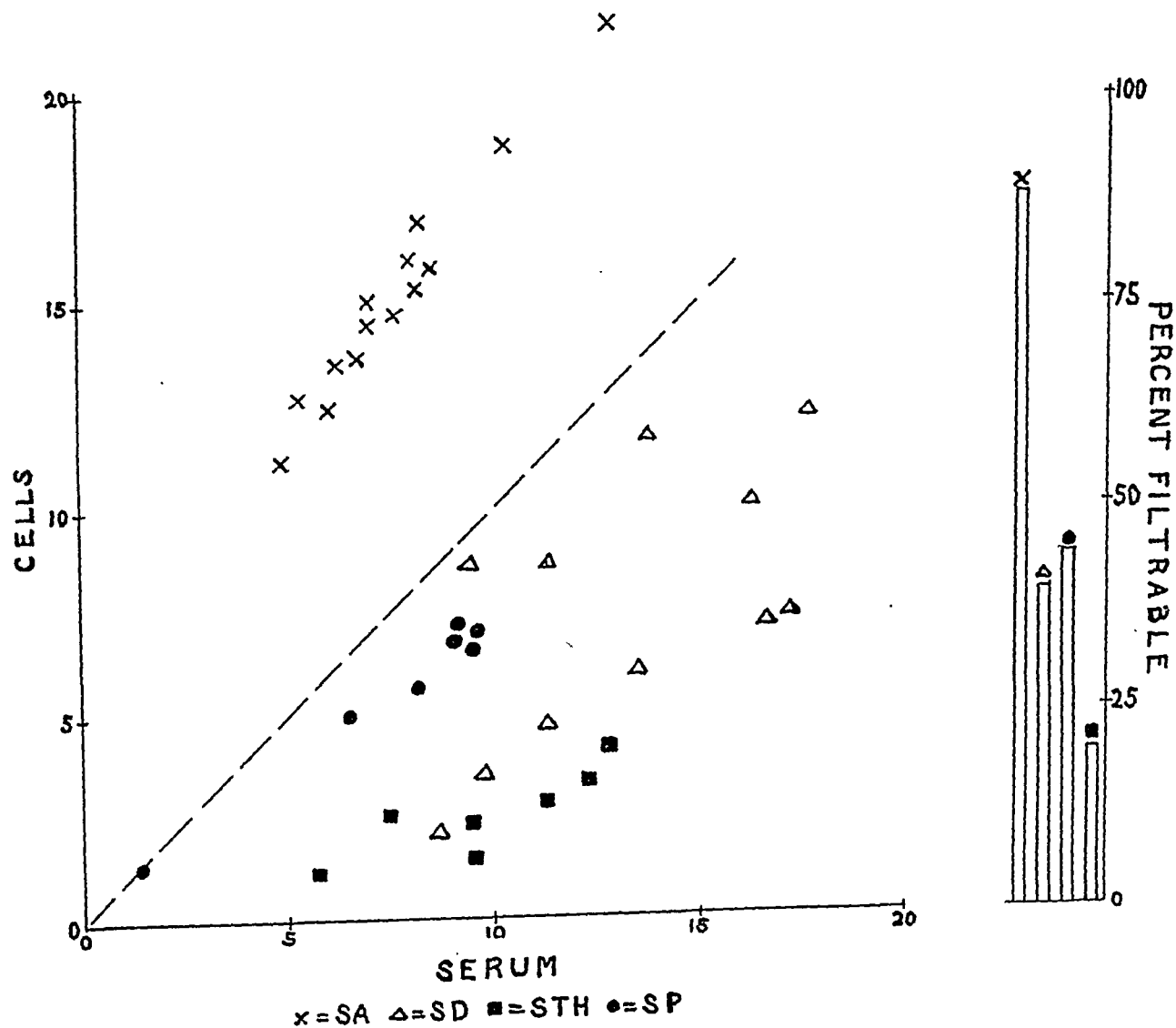


FIG. 1. CONCENTRATIONS OF SULFANILAMIDE, SULFAPYRIDINE, SULFADIAZENE, AND SULFATHIAZOLE, EXPRESSED AS MGM. IN 100 CC. OF WATER IN CELLS AND SERUM.

On the right hand side are represented the concentrations in serum ultrafiltrates, expressed as per cent of the original serum concentrations.

The observation that a substance like sulfanilamide passes freely in both directions (cells  $\rightleftharpoons$  serum) principally excludes its use for measurements of body water. Results of experiments conducted on dogs by Elkinton and Taffel (11) have failed to corroborate a report by Painter (12) that sulfanilamide is suitable for the measurement of body fluids.

Our observations that varying percentages of the different drugs are ultrafiltrable from serum, confirm Davis' (13) report that similar proportions are diffusible when plasma is dialyzed against saline. Schoenholzer (14) and Davis (13) concluded that sulfonamides were bound to the albumin fraction of plasma protein. In this connection, it is noteworthy that the rate of diffusion of sulfonamide compounds through agar and gelatine gels was found by Hawking (15) to decrease in this order: sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazene.

The free exchange of these substances between the two phases of blood, and the varying proportions of nonfiltrable drug in plasma, demonstrate the necessity of interpreting studies on clearances of these compounds with great reserve. It is obvious that such studies cannot be based on concentrations in whole blood. In *in vivo* experiments, such as observations on clearances, an additional fallacy may arise from difficulties which were noted while analyzing these drugs in urine. Determinations in urine to which nonacetylated drugs had been added yielded "acetylation" up to 15 per cent, which is far greater than the error of the method. Studying simultaneously original urine and urine autoclaved at 250° F. for 30 minutes, as well as urine passed through a Seitz filter, identical proportions of acetylation were observed. Active enzymes or the presence of bacteria, therefore, can not be responsible for this change which seems to be an artefact. Urine from the same person behaved differently on different days, a change which could not be attributed to differences in pH. It seems at present that for experimental purposes measurements of these substances in urine are not sufficiently reliable.

The amount of drug bound to plasma protein appears to be bacteriostatically ineffective. On the basis of this finding by Davis (13), and of the observation of unequal distribution, measurements

of these compounds in whole blood must be considered to be less informative than measurements in serum or plasma or ultrafiltrate. With whole blood concentrations of 11 mgm. per cent for sulfathiazole and for sulfanilamide, for example, the serum concentration of the former was 16.5, and that of the latter, 10.2 mgm. per cent. The concentration of ultrafiltrable sulfathiazole, not bound to plasma protein, would be 3.3, and that of sulfanilamide, 8 mgm. per cent.

The ratios of distribution noted under the experimental conditions described are in agreement with observations reported by others (6 to 10).

Figure 1 demonstrates that the concentrations in cells vary with the percentage of ultrafiltrable substance in serum. The greater this percentage, the more seems available for passage into the cells. The proportion of drug bound to serum protein, therefore, appears to determine the ratio of distribution of these compounds. Such an extracellular factor appears the more likely since it could be shown that the transfers occur from serum to cells as well as from cells to serum; an intracellular reaction, like that displayed by ascorbic acid, therefore, can hardly be responsible for the concentrations of sulfonamides attained in cells.

#### SUMMARY AND CONCLUSIONS

Sulfanilamide, sulfathiazole, sulfapyridine, and sulfadiazene were measured colorimetrically after addition to defibrinated human blood.

The reaction associated with the distribution of these substances is instantaneous, and independent of temperature and the presence of oxygen.

Transfer of these compounds occurs, under suitable experimental conditions in both directions, from serum to cells and from cells to serum. Sulfonamide compounds attain equal concentrations in erythrocytes and in leukocytes.

The percentages of these drugs in serum ultrafiltrates vary; they correspond to the concentrations they attain in cells and seem to determine their rate of distribution in whole blood. Of the four compounds studied, only sulfanilamide was found to be more concentrated in cells than in serum; sulfathiazole, on the other hand, reached the highest relative concentration in serum.

Free passage of these compounds and varying ratios of distribution suggest that measurements



of concentrations in plasma or in serum would be more informative than in whole blood. These observations also exclude the use of any of these substances for measurements of body fluids.

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# OBSERVATIONS ON CERTAIN MANIFESTATIONS OF CIRCULATORY CONGESTION PRODUCED IN DOGS BY RAPID INFUSION

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(Received for publication June 11, 1942)

The intravenous injection of fluids has been frequently utilized to study the dynamics of the circulation. The purpose of this study is to describe the response of intact dogs to such injections. In a previous paper (Swank, Porter and Yeomans (1)), the infusion method described in this paper was used to study changes in the adaptability of the cardiovascular system in thiamin deficient dogs.

## METHODS

The following experimental procedure was finally adopted. Non-pregnant female dogs, weighing between 10 and 18 kgm., were used. After fasting 18 to 24 hours, the animal to be studied was anesthetized with an intravenous solution of alpha chloralose (100 to 110 mgm. per kgm. of body weight) or pentobarbital sodium (33 mgm. per kgm.), and placed on its back on a dog board, with its legs lightly secured. Number 18 Lindeman needles were placed in one jugular and one femoral vein, and a glass catheter was placed in the bladder. The needle in the femoral vein was attached, by a side-arm syringe and rubber tubing, to a copper coil kept at a constant temperature by a water bath. The infusion fluid flowed through this coil from a glass reservoir which could be read accurately to 10 cc. The speed of flow of the infusion was regulated by raising or lowering the reservoir. The needle in the jugular vein was attached to a manometer containing  $\frac{1}{6}$  molar sodium carbonate solution. This solution was allowed to run into the jugular needle at the rate of approximately 4 to 5 cc. per hour, as described by Trendelenburg (2). This prevented the blood from clotting and allowed the venous pressure to be accurately indicated throughout the experiment. Since it would have been necessary to sacrifice each animal to determine accurately the level of the right auricle, the back of the animal was arbitrarily chosen as the zero-point for venous pressure. This seemed sufficient for our purpose as we were interested in comparing the results of different infusions in the same animal, and in changes in venous pressure rather than in absolute values. A rubber tube of 5 mm. internal diameter was placed in the dog's trachea to assure a free air passage.

After the venous pressure and heart rate had been recorded several times, the infusion was begun and run for 30 minutes by stop watch, at the rate of 2.5 to 5.0

cc. per kgm. per minute. At 2 minute intervals during<sup>1</sup> the infusion, the jugular venous pressure, the heart rate, and the amount of the infusion were read and recorded. The urine output was measured at 10 minute intervals. After the infusion was discontinued, venous pressure, cardiac rate, and urine output were observed until the venous pressure returned to, or nearly to, normal. The length of the infusion period and its volume were chosen because they were found to approach the limit of tolerance in healthy dogs. Moreover, this period allowed the venous pressure in normal dogs to become adjusted to the increased plasma volume. With alpha chloralose, the anesthesia was quite satisfactory throughout the entire experiment, whereas with pentobarbital sodium it was frequently necessary to give additional anesthetic toward the end of the infusion.

Arterial pressures in mm. Hg, when taken, were determined directly, by inserting a needle into the femoral artery. Clotting in the needle was avoided by allowing a small amount of  $\frac{1}{6}$  molar sodium carbonate to run into the artery continuously (2).

During some of the infusion experiments the following supplementary data were obtained:

(1) The oxygen consumption was determined with a Benedict-Roth apparatus. This was connected to the dog by means of a Bialock mask (3).

(2) Measurements of the cardiac output were obtained by means of the Fick formula. Blood was drawn under oil simultaneously from the right heart and a femoral artery. The heart was auscultated during this procedure and gross changes in its rate were noted. The oxygen content and capacity of the blood samples were immediately determined in duplicate by the method of Van Slyke and Neill (4).

(3) Plasma volume was measured by the method of Gibson and Evans (5) using the azo dye, Evans Blue, and the spectrophotometer.

(4) Variations in cardiac rhythm and sounds were noted by auscultation, and in suitable cases recorded by electrocardiograms or phonocardiograms.

(5) Changes in the heart size during infusion were determined by repeated x-rays using a standard distance of 6 feet.

<sup>1</sup> The advantage in accuracy, of avoiding interruption of infusion while reading pressure, which the use of a separate vein provides, may be seen in Figure 1. Discontinuance of infusion for one minute at A and B caused a large fall in pressure.

TABLE II

*Comparison of jugular and portal venous pressures during saline infusions*

Anesthesia	Abdomen open		Extraperitonealized spleen	
	Pentobarbital-sodium		Alpha chloralose	
Rate of infusion	3.8 cc. per kgm. per minute		4.5 cc. per kgm. per minute	
Time	Jugular venous pressure	Portal venous pressure	Jugular venous pressure	Portal venous pressure
minutes	mm. H <sub>2</sub> O	mm. H <sub>2</sub> O	mm. H <sub>2</sub> O	mm. H <sub>2</sub> O
0	157	194	138	280
2	165	198	163	298
4	167	204	188	307
6	168	213	197	313
8	168	221	225	320
10	168*	230*	250	335
12			260	335
13	170	243		
14			275	353
15	171	270		
16			288	357
17	179	280		
18			297	357
19	184	302		
20			308	373
21	183*	302*		
22			324	388
24	190	301	330	398
26	187	300	340	413
28	189	300	340	408
30	196	303	335†	408†
31			290	361.
32	†	†		
33			258	338
35			237	328
38½	162	269		
41	157	262	202	300
45			193	282

\* Infusion stopped for 1 minute.

† Infusion completed.

ally necessary to interrupt dextrose-in-saline infusions because of the very alarming venous pressures which were apt to develop. The indication for caution in therapeutic use of this hypertonic solution is evident.

**Portal pressures.** The data concerning pressures in the portal system during infusion were obtained from 2 experiments (Table II). In each of these, both the initial and final portal

venous pressures were higher than the initial or final peripheral venous pressures, although, in one instance, the portal pressure rose more than the systemic venous pressure, and in the other, it rose somewhat less. A high portal pressure was also evidenced by the development of a marked swelling of the abdomen and enlargement of the liver during all infusions, and swelling of the spleen in the 2 cases in which the size of this organ could be determined.

TABLE III

*Increase in plasma volume and output of urine during and after infusion*

Infusing fluid	Time from start of infusion	Plasma volume	Increase in plasma volume	Urine output	Amount infusion fluid	Rate of infusion
	minutes	cc.	per cent	cc.	cc.	cc. per minute per unit initial plasma volume
Dog 10—July 31, 1939. Normal saline	0	855				7.9
	10	1370	60.2	0	727	
	20	1405	64.3	47	1407	
	30*	1820	112.8	62	2090	
	40	1290	50.9	105		
	50	1350	57.9	80		
Dog 10—August 3, 1939. 10 per cent dextrose in saline	0	958				7.3
	10	2010	109.8	162	700	
	20	2530	164.0	180	1400	
	30*	2600	171.5	260	2100	
	40	1910	99.4	300		
	50	1270	32.6	340		
	55			148		
	65			185		
Dog 14—August 2, 1939. Normal saline	0	670				9.7
	10	965	44.0	0	645	
	20	1280	91.1	4	1310	
	30*	1650	146.2	95	1950	
	40	1072	60.0	160		
	50	875	30.6	170		
	55			95		
Dog 14—August 4, 1939. 10 per cent dextrose in saline	0	755				8.6
	10	1775	135.0	245	650	
	20	2700	257.5	490	1300	
	30*	2830	275.0	440	1950	
	40	1490	97.4	440		
	50	965	27.8	258		
	55			110		

\* End of infusion, which was interrupted for one minute at 10 and 20 minutes.

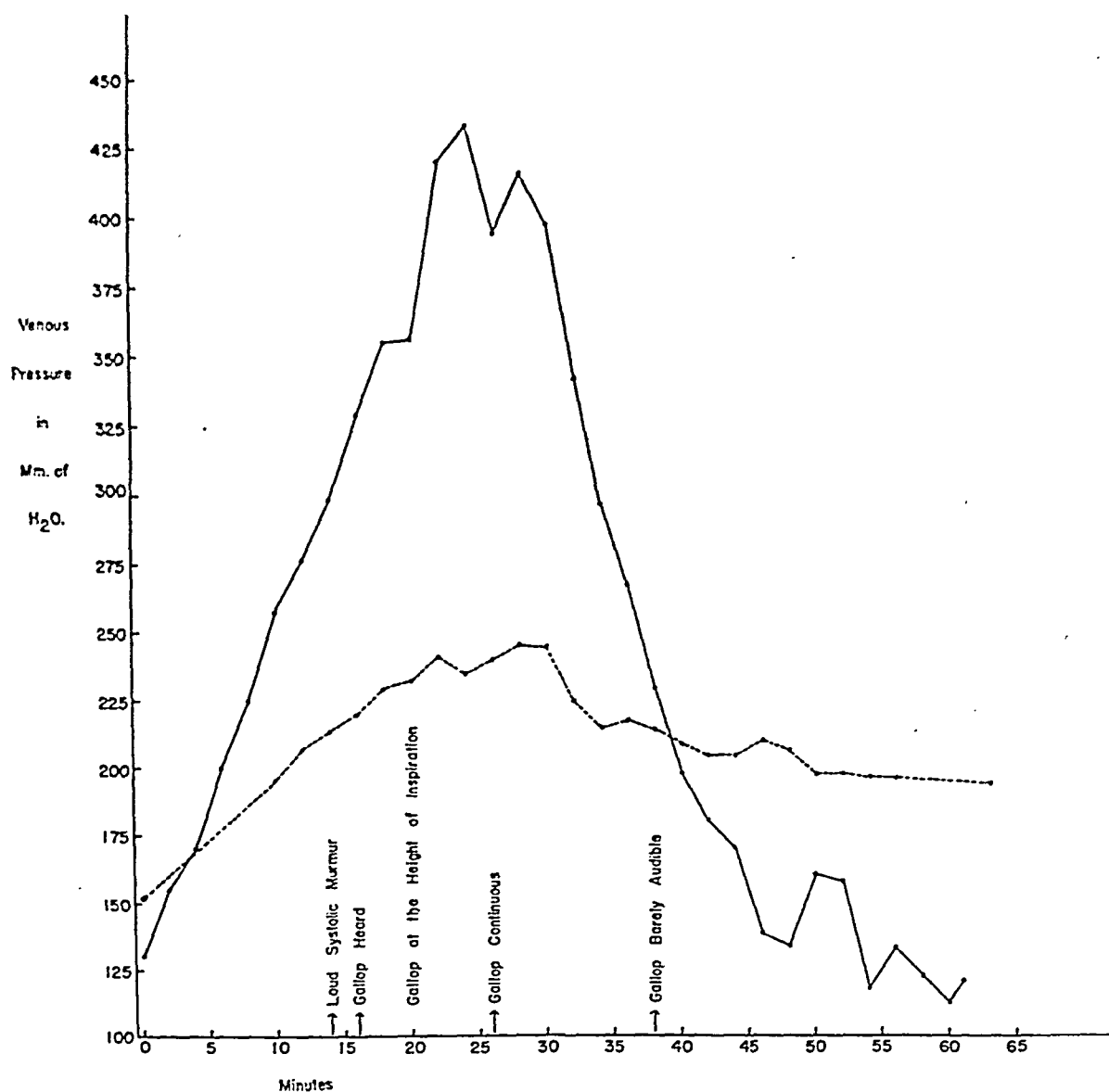


FIG. 5. MEASUREMENTS OF LEFT AURICULAR PRESSURE AND PERIPHERAL VENOUS PRESSURE

Continuous line represents the left auricular pressure. Interrupted line represents the femoral venous pressure. Time of infusion is 30 minutes.

*Pulmonary venous pressure.*<sup>3</sup> The effect of infusion on the pulmonary circuit was studied in one experiment (Figure 5). Under alpha chloralose anesthesia, a rubber catheter was stitched into the left auricular appendage and the usual manometer attached. After the left lung had been re-expanded, an infusion of saline was given, and the peripheral venous and left auricular pressures

<sup>3</sup> We are indebted to Dr. Robert Gross for the surgical part of this experiment.

were recorded. Before the infusion, the auricular pressure rose and fell some 80 mm. with expiration and inspiration, respectively. After the auricular pressure had risen as a result of infusion, these marked respiratory excursions gradually decreased, but appeared again, following the cessation of infusion. The graph of this experiment shows that the pressure in the pulmonary circuit rose more rapidly and to a much higher level than was observed in the peripheral venous circuit dur-

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	30*	1650	146.2	95	1950	
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	50	875	30.6	170		
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# EXPERIMENTAL CIRCULATORY CONGESTION BY RAPID INFUSION

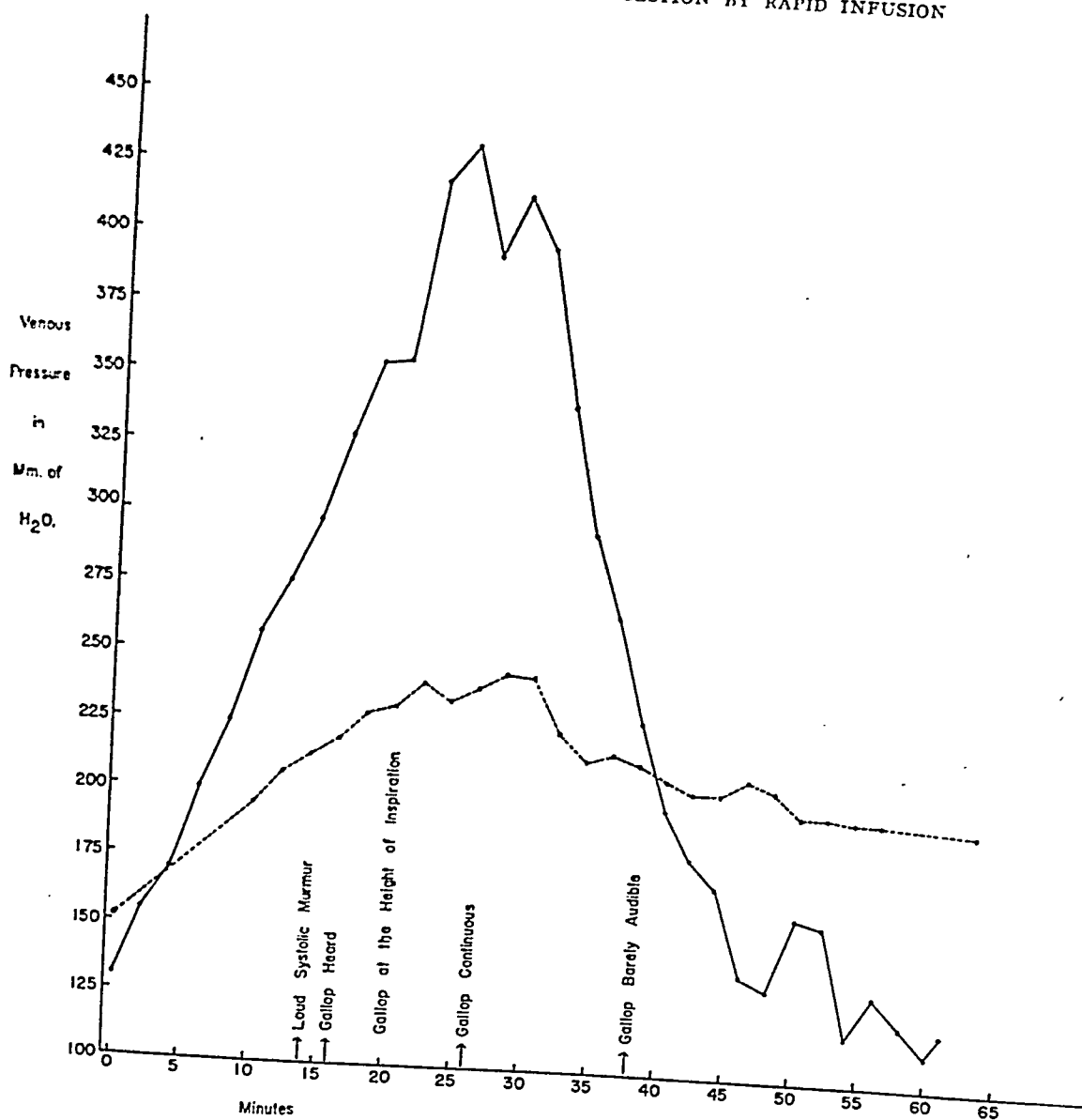


FIG. 5. MEASUREMENTS OF LEFT AURICULAR PRESSURE AND PERIPHERAL VENOUS PRESSURE  
Continuous line represents the left auricular pressure. Interrupted line represents the femoral venous pressure.  
Time of infusion is 30 minutes.

*Pulmonary venous pressure.*<sup>3</sup> The effect of infusion on the pulmonary circuit was studied in one experiment (Figure 5). Under alpha chloralose anesthesia, a rubber catheter was stitched into the left auricular appendage and the usual manometer attached. After the left lung had been re-expanded, an infusion of saline was given, and the peripheral venous and left auricular pressures

<sup>3</sup> We are indebted to Dr. Robert Gross for the surgical part of this experiment.

were recorded. Before the infusion, the auricular pressure rose and fell some 80 mm. with expiration and inspiration, respectively. After the infusion, the pressure had risen as a result of infusion, these marked respiratory excursions gradually decreased, but appeared again, following the cessation of infusion. The graph of this experiment shows that the pressure in the pulmonary circuit rose more rapidly and to a much higher level than was observed in the peripheral venous circuit dur-

ing infusions of the same speed. In this experiment, no pulmonary râles were heard, but a systolic murmur and a gallop rhythm appeared.

*Plasma volume:*<sup>4</sup> Representative studies of the plasma volume changes during infusion are shown in Table III and Figure 6. Calculations of the total blood volume are not recorded, as the red blood cell volume during and after infusion fluctuated markedly, probably because of an uneven dilution of the red blood cells which occurred during the infusion.

The infusions of 10 per cent dextrose in saline not only produced a much greater increase in plasma volume than did saline alone, but did so even when the infusions were slower. Apparently this very hypertonic solution caused fluid to be drawn into the blood stream from outside the vascular system in addition to that injected, since in dog 14 the total amount of injected fluid at the termination of infusion was less than the fluid

which had been added to the initial plasma volume, and in dog 10, an addition to the plasma volume of 1572 cc. had taken place, whereas but 1400 cc. of fluid had been injected at the twentieth minute of infusion. Another indication of this very marked increase in the blood volume is to be found in the alarming rise in venous pressure, mentioned before. Both during and after 10 per cent glucose in saline infusions, a tremendous diuresis occurred. Usually, the plasma volume returned to normal more rapidly after glucose than after saline injections.

*Serum proteins.* The total serum proteins were decreased by all infusions (see Table IV). At rates of flow between 55 and 70 cc. per minute, the total protein content of the serum was almost halved. The non-protein nitrogen also fell during infusions, but less than the proteins.

*Heart rate.* In dogs anesthetized with pentobarbital sodium, the heart rate was usually rapid and quite variable, both before and during infusion. The sinus arrhythmia was never marked. There was a tendency, however, for the rate to

<sup>4</sup> We wish to express our appreciation to Dr. John G. Gibson, II, for his aid in these studies.

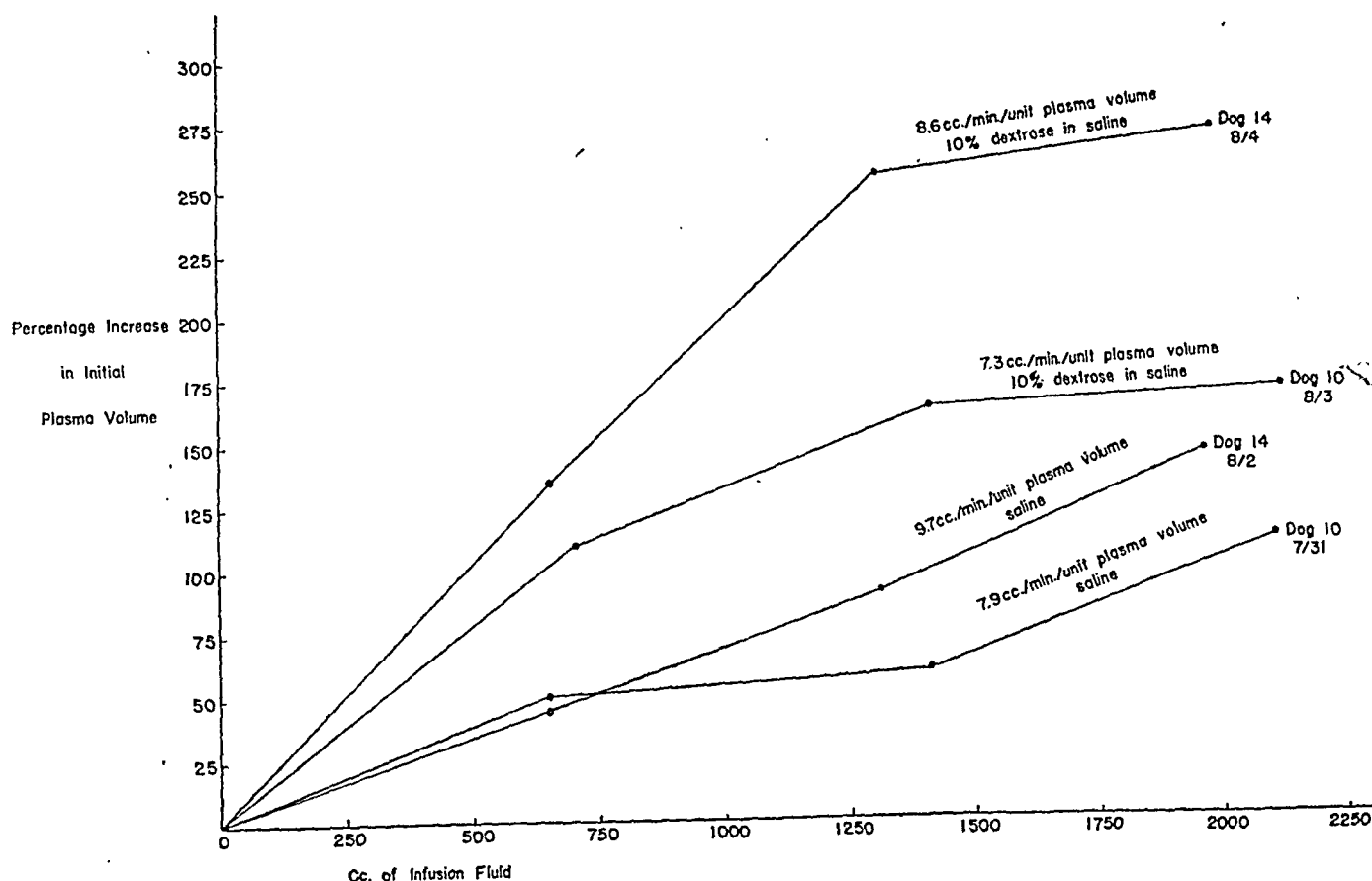


FIG. 6. CHANGES IN THE PLASMA VOLUME OCCURRING AS A RESULT OF INTRAVENOUS INFUSIONS WITH DEXTROSE IN SALINE AND WITH SALINE ALONE

TABLE IV  
Effect of infusion upon the serum proteins in dogs

Dog number	Serum protein		Non-protein nitrogen		Rate of infusion	Weight
	Before infusion	After infusion	Before infusion	After infusion		
	grams per 100 cc.		mgm. per 100 cc.		cc. per minute	kgm.
17	5.2	3.3	20	14	55	12.2
10	5.8	3.0	41	31	65	18.8
10	5.2	3.1	34	25	70	18.8
15	6.0	3.1	40	30	62	18.4
15	6.1	3.1			60	18.4

increase toward the termination of infusion and sometimes it exceeded 200 beats a minute.

With chloralose, the heart rate was more often relatively slow preceding the infusion, and the sinus arrhythmia, normally marked in dogs, was unchanged. In the majority of experiments, the heart rate increased within the first 2 minutes of injection, and continued to increase throughout the infusion, to reach a rate approximately 40 to 70 beats faster than was observed before infusion.

**Arterial blood pressure.** The recording of the mean arterial blood pressure, during 5 infusions on 4 animals, showed a rise of 10 to 20 mm. of mercury during the latter part of the infusion, in

4 instances. During 3 injections, this rise was preceded during the first 16 minutes by a slight temporary fall (about 10 mm. of mercury) in the mean pressure.

**Cardiac output.** The cardiac output was determined, during infusion on 17 occasions, in 6 normal dogs under chloralose anesthesia. In Figure 7, the increase in cardiac output is plotted against the duration of the infusion. This demonstrates that the greatest increase in cardiac output occurred during the first few minutes of the infusion. In 2 instances, it was shown by successive cardiac outputs taken during the same infusion that there was a slight further increase in cardiac output during the latter part of the infusion period. Isolated studies on different dogs on different days (Figure 8) showed that the cardiac output increased to a variable degree throughout the infusion, and was in no clear way related to the height of the venous pressure. This increased cardiac output persisted for as long as 10 minutes after the completion of the infusion, although the venous pressure had returned nearly to normal by that time.

**Arrhythmias and murmurs during infusion.** During infusion, there was a disappearance of the

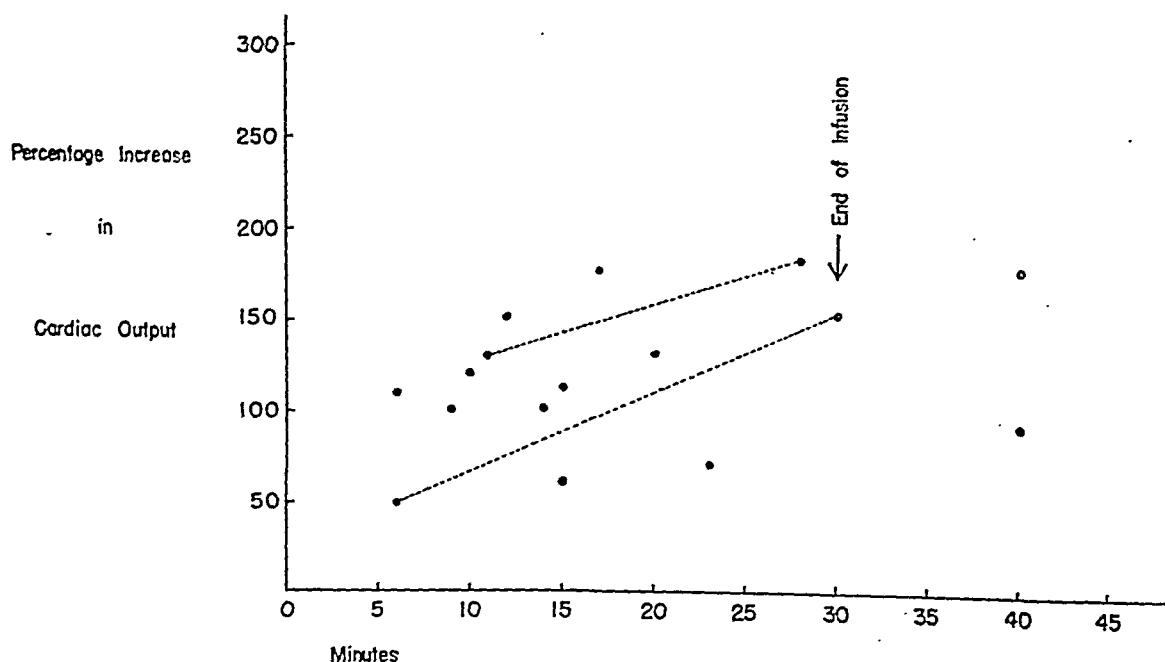


FIG. 7. THE PERCENTAGE INCREASE IN THE OUTPUT OF THE HEART WITH INTRAVENOUS INFUSION  
The interrupted lines connect measurements made on the same animal at two points during one infusion.



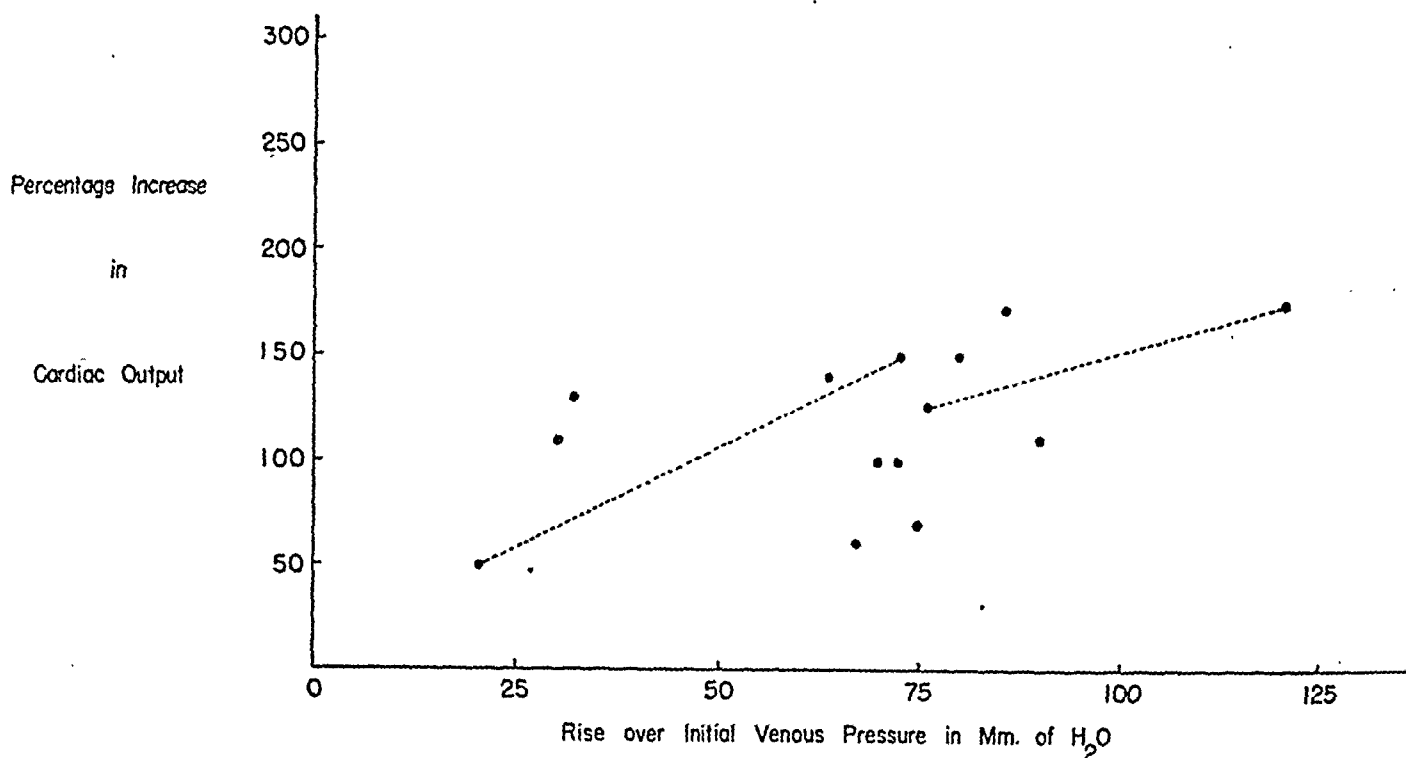


FIG. 8. REPRESENTS THE RELATION BETWEEN THE INCREASE OF VENOUS PRESSURE OCCURRING DURING INFUSION AND THE PERCENTAGE INCREASE IN CARDIAC OUTPUT

Interrupted lines connect measurements made on the same animal at two points during one infusion.

sinus arrhythmia normally present in alpha chloralose anesthetized dogs. Extrasystoles were noted only once during infusion.

In 4 dogs that had each received 7 to 15 infusion tests, systolic murmurs were observed during basal conditions. This suggests that the heart had become affected permanently by this procedure. A systolic murmur, usually heard best to the left of the sternum, almost always developed during an infusion. Although the venous pressure was always elevated to some degree when the murmur appeared, it could not be correlated with any specific elevation in this pressure. Neither could it be correlated with a rise in heart rate, since in some experiments with pentobarbital sodium, the murmur appeared when the cardiac rate was slower than before infusion. During the recovery period, the murmur usually disappeared at about the level of venous pressure where it was first noted during the infusion. Despite this relationship, it is probable that the murmur is more directly associated with changes in the pulmonary than in the peripheral venous pressures.

Usually, during the second 10 minutes of infusion and shortly after the appearance of the systolic murmur, a gallop rhythm was noted. The additional third sound was first audible during the

height of inspirations and soon after throughout the respiratory cycle. By means of a phonocardiogram, with simultaneous jugular pulse and electrocardiogram, the presence of this extra sound was clearly established (Figure 9). Careful examination of the control record (A) reveals that no sound was recorded during diastole just before the occurrence of the first heart sound. During the infusion, when a gallop rhythm could be clearly auscultated (B), a new sound was recorded, just before the first heart tone. This seems to identify the gallop rhythm as presystolic in time.

No relationship was found between heart rate and the appearance of the gallop rhythm in dogs anesthetized by pentobarbital sodium. Indeed, in 11 of a series of 19 infusions, the heart rate was below the preinfusion rate at the time the gallop rhythm first became audible. The slowest heart rate associated with a gallop rhythm in pentobarbital anesthetized dogs was 112 beats per minute, the most rapid, 204 beats per minute. In these same animals, the venous pressure was elevated between 20 and 130 mm. of water. With alpha chloralose as an anesthetic, the gallop rhythm was noted only when the heart rate was increased over basal levels, *e.g.*, to between 134 and 180 beats per minute, and usually, when the venous

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pressure was increased 25 to 90 mm. of water. In general, it may be said that when the gallop rhythm appeared with a heart rate in the lower range, the venous pressure was more frequently in the upper range; when the heart rate was in the upper range, the venous pressure was in the lower range. With

both anesthetics, it is probable that the gallop rhythm, like the systolic murmur, was related more directly to changes in the pulmonary than in the peripheral venous pressure.

*Heart size during infusion.* X-rays of the heart during infusion were made in 4 instances on 2

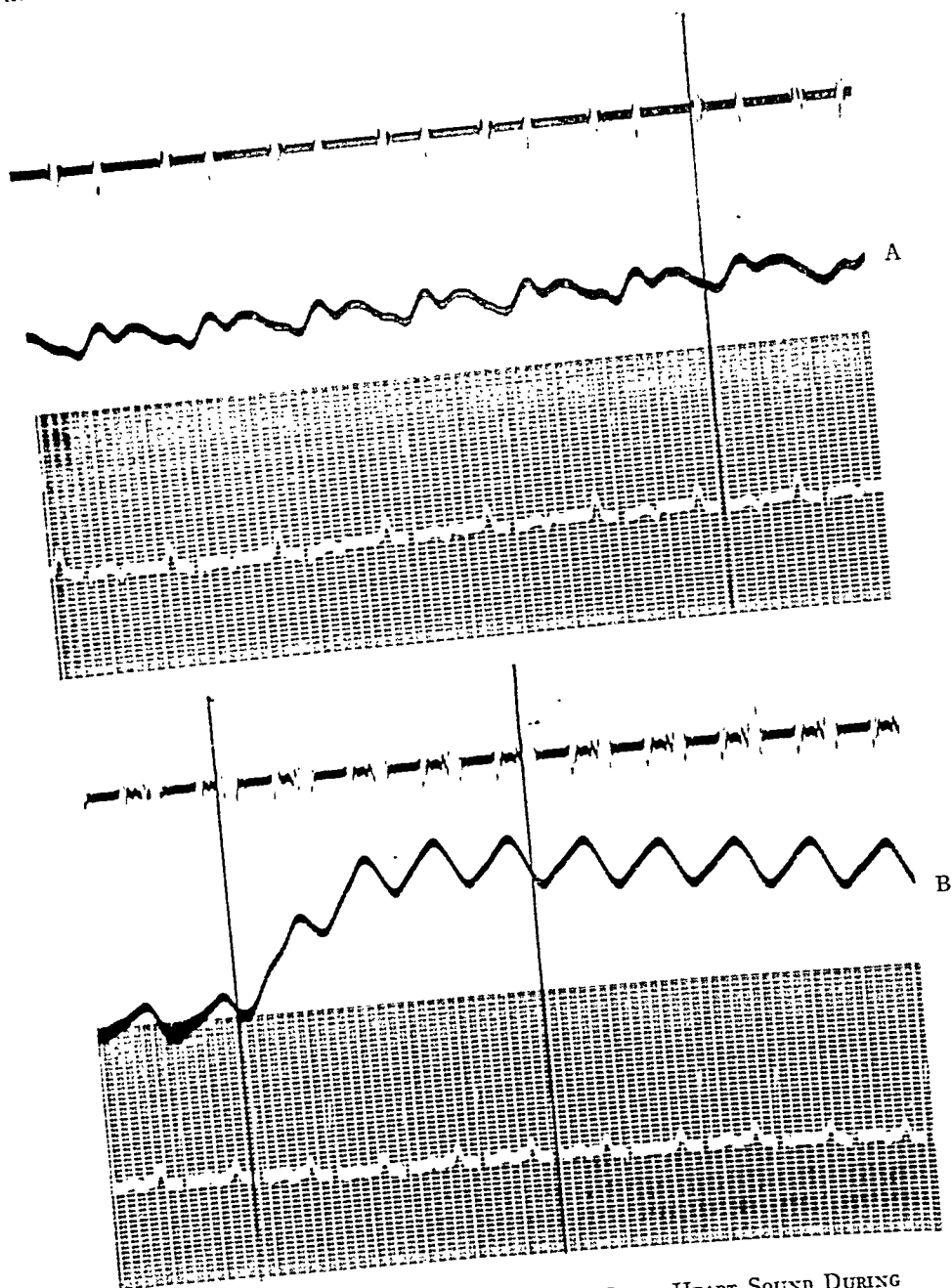


FIG. 9. REPRESENTS THE APPEARANCE OF A THIRD HEART SOUND DURING AN INTRAVENOUS INFUSION

A is normal control, B, during infusion.

dogs. In 2 of the experiments, the anesthetic was pentobarbital sodium, and in 2 others, alpha chloralose. There was a progressive increase in heart diameter during infusion, the greatest being 1 cm. The diameter of the heart appeared to increase more rapidly and to a greater extent under pentobarbital sodium, but this could not be substantiated by careful measurements of the circumference of the heart.

*Oxygen consumption during infusion.* The oxygen consumption during an infusion generally increased somewhat, the amount of increase in the majority of instances varying between +1 and +13 per cent. The apparent vigor of the heart action was increased, and respiration was accelerated, although slightly diminished in depth. These heightened activities probably account in part for

the moderate increase in oxygen consumption noted in some experiments.

## DISCUSSION

By rapidly increasing the plasma volume, many of the phenomena observed in patients with congestive heart failure (*e.g.*, a rise in peripheral venous pressure, an increase in the heart size and rate, the development of a systolic murmur and gallop rhythm, and swelling of the abdomen, liver, and spleen) were produced in the dog. It is significant that our dogs differed from most patients with congestive heart failure in their ability (*a*) to maintain a high cardiac output and (*b*) to prevent the venous pressure from rising above a critical level; this, in spite of the fact that the plasma volume had increased very much more than it does

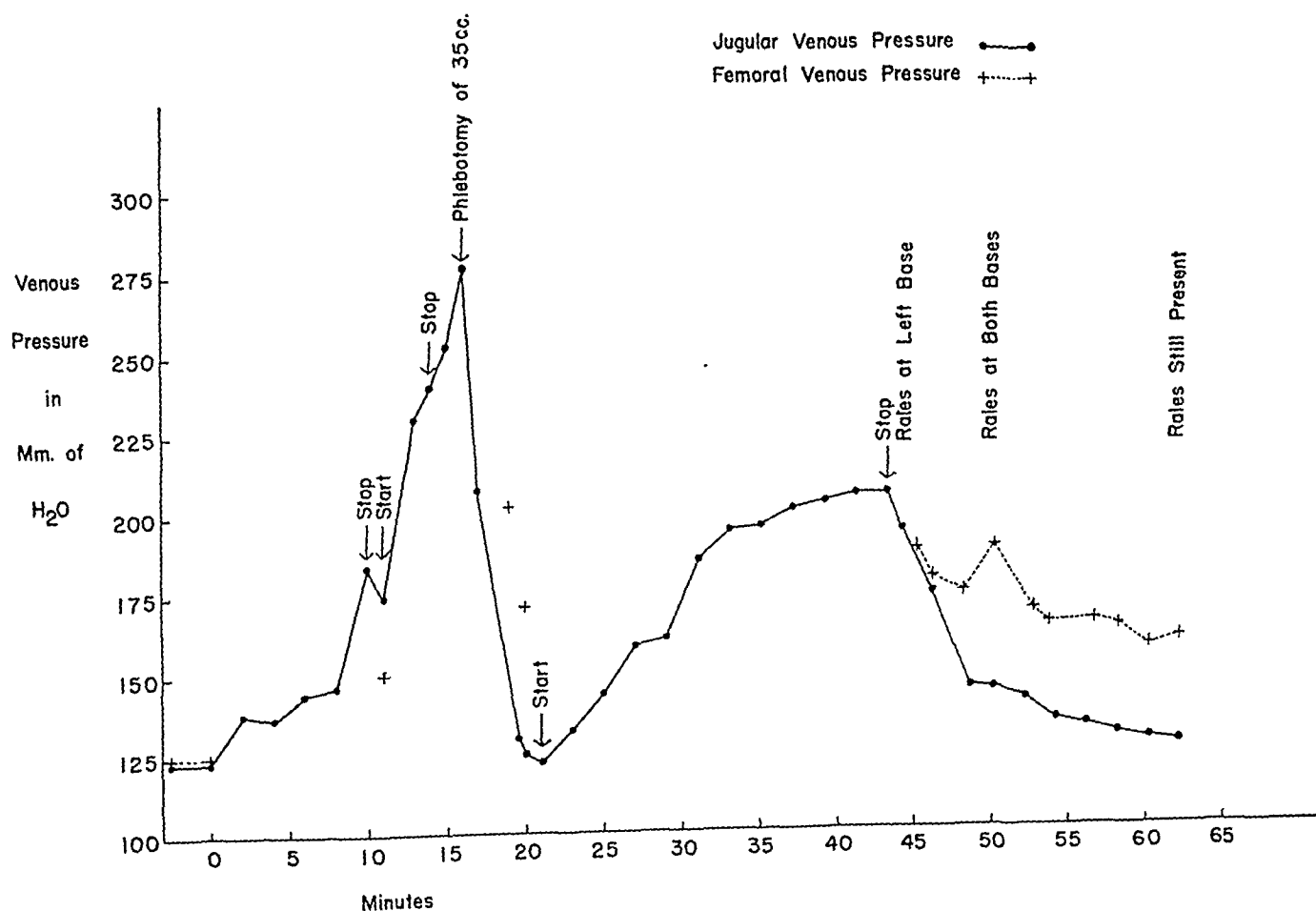


FIG. 10. THE EFFECT OF A PHLEBOTOMY CARRIED OUT DURING INFUSION WITH 10 PER CENT DEXTROSE IN NORMAL SALINE

This was performed as emergency treatment because of rapidly rising peripheral venous pressure. In spite of the rapid fall of pressure to normal following phlebotomy and lower curve when the infusion was again continued, evidences of high pressure in the pulmonary circuit developed. The arrows marked "stop" and "start" indicate temporary interruptions of the infusion. Rate of infusion during the first 14 minutes was 4.2 cc. per kgm. per minute. Afterwards it was continued at 3.3. cc. per kgm. per minute.

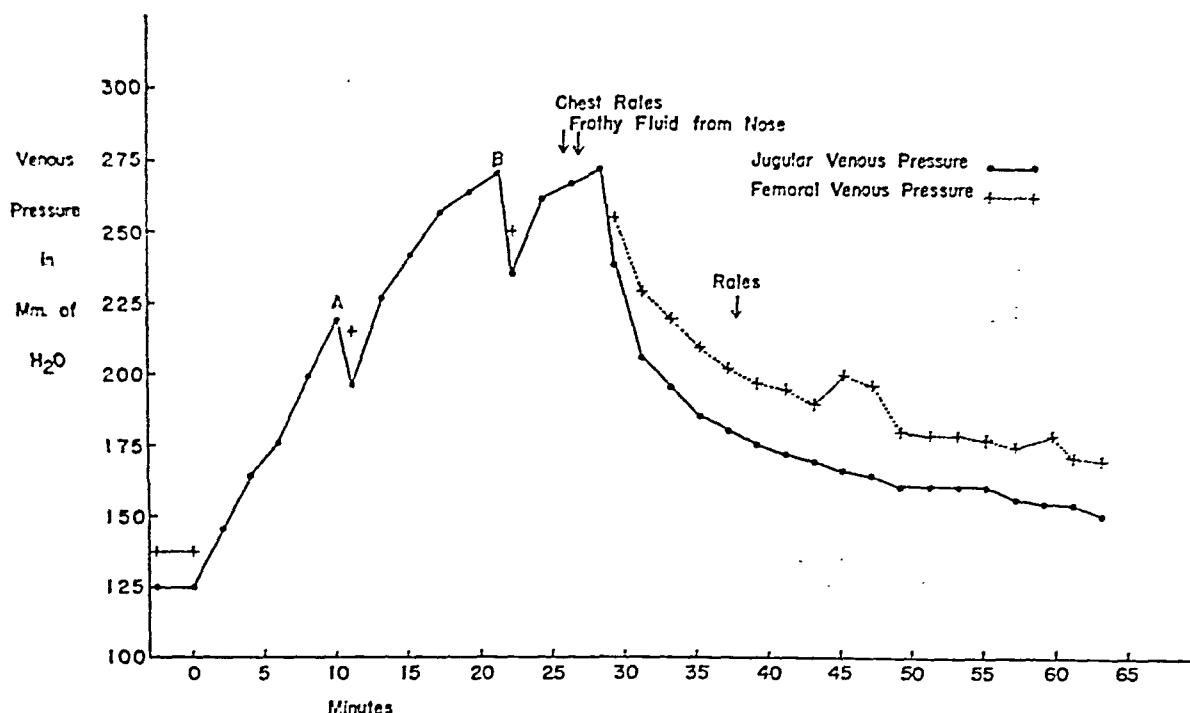


FIG. 11. REPRESENTS INFUSION WITH 10 PER CENT DEXTROSE IN SALINE IN A NORMAL DOG

Although this curve is high, it is not unlike other curves obtained with 10 per cent dextrose in saline, in other animals which showed no signs of failure of the left ventricle.

in patients with cardiac failure. In this connection, it is impossible to overstate the importance of a healthy and vigorous heart.

Of considerable interest was the stabilization of the venous pressure during the later part of the experiment. This evidently indicates adjustment of the cardiovascular system to a greatly increased blood volume. The following and perhaps other factors were jointly responsible for this: (1) a continued increase in the cardiac output, (2) an increase in the fluid removed from the vascular system, and (3) an increase in the capacity of the vascular system. The cardiac output probably played a relatively small part, since by far its greatest increase occurred early, and while the venous pressure was still increasing rapidly. On the other hand, during the latter part of some infusions, especially when glucose solutions were used (see Figure 6), it was evident from blood volume studies that fluid was being lost from the intravascular system by way of the kidneys or into the tissues of the body at a very rapid rate. In these experiments, the loss of fluid must have been instrumental in maintaining the

venous pressure at a fairly constant level. In others, an increase in the capacity of the vascular system must have been equally effective, since the plasma volume continued to increase steadily. Evidences of this increased capacity were to be found in the dilated peripheral, portal, and pulmonary venous channels, the increased size of the abdomen, liver, spleen, and heart, and congestion of the lungs. Dilatation of the peripheral capillary system, which occurs soon after an infusion is started (6, 7), must also have played a role. It should not be assumed from this discussion, however, that the increased venous pressure was anything but helpful, provided it did not rise too high. No doubt this increased the cardiac output and helped to maintain an appropriate distribution of blood.

Whenever, in these experiments, a rapid elevation of peripheral venous pressure occurred during the period when this pressure was usually stable, or pulmonary râles developed (Figures 10 and 11), one must assume that the ability of the cardiovascular system to adjust itself to its increased load had been exceeded, and that the elevated

venous pressure which was previously helpful, now by virtue of an overdistention of the cardiovascular system, constituted a menace. This may be considered in some degree a counterpart of cardiac failure in humans, but certain obvious qualifications need be mentioned. First, the plasma volume was very rapidly increased in our dogs, whereas it is probable that, in humans with cardiac failure, the plasma volume increases much more slowly. Theoretically, it would seem that such a sudden change would produce a maximum stimulation of the heart and at the same time allow a minimum opportunity for an increase and adjustment in the capacity of the vascular system. Second, the concentration of both the plasma proteins and some electrolytes was greatly reduced by this sudden addition of fluid to the blood (8), thus lowering its osmotic pressure, and facilitating the production of pulmonary râles and indistinguishable edema elsewhere. It is interesting to note that pulmonary edema was more apt to develop during glucose infusions, even though this hypertonic solution drew fluid into the vascular system from the interstitial tissues. Apparently, the greatly increased blood volume elevated the hydrostatic pulmonary pressure sufficiently to overcome the osmotic pressure. This would seem to point to the presence of a very high pulmonary capillary pressure in these experiments.

Probably our most interesting observations concerned the pressure relationships which developed during infusion in the general, portal, and pulmonary venous systems. Notably the pressure in the left auricle, which preceding infusion was lower than the general venous pressure, rose very rapidly and to a much higher level than did the general venous pressure. Following infusion, it also fell to normal much more rapidly. This would seem to indicate that the resistance to the flow of blood through the lungs became enormously reduced during infusion, thus allowing a large part of the pulmonary arterial pressure to be transmitted to the venous side of the circuit. Within limits, this increased left auricular pressure would be helpful by increasing the cardiac output. Too great a rise in pressure would eventually lead to pulmonary edema and (unless the infusion were stopped) death. Figures 10 and 11 show two instances in which pulmonary edema occurred in the absence

of a greater than usual increase in the peripheral venous pressure. In both of these instances, glucose infusions were used, but this observation was true of saline infusions as well. It is of considerable interest that the pressure in the left auricle can rise so high (Figure 5) without the occurrence of pulmonary râles. In a recent paper dealing with left heart failure in patients, Burwell (9) discussed this same experiment, pointing out how independent of one another the left and right sides of the heart can be in patients.

The portal pressure in two instances was higher than the preinfusion general venous pressure, and rose to a level intermediate between the general venous and left auricular pressures. Two anatomical facts are of interest in this connection. First, the portal system veins have very thick muscular walls, capable, it would seem, of withstanding a considerable increase in pressure. Second, in dogs, circular muscular bands are present in the walls of the hepatic veins. These are so disposed as to be theoretically capable of checking the flow of blood (10), and consequently could effect a storage of blood in the portal system. In fact, this seems to have occurred during infusion since in addition to a marked elevation of the portal pressure, and increase in the size of the spleen in two cases, in all cases marked swelling of the liver and of the abdomen was observed. These facts seem to indicate that the portal, as well as the pulmonary system contained more than its share of the increased plasma volume during infusions. A similar mechanism could operate in patients with congestive heart failure.

#### SUMMARY

Rapid infusion in dogs produced:

- (1) Congestion in the peripheral, pulmonary, and portal venous systems, evidenced by rises in their venous pressures, swelling of the abdomen, liver, and spleen, and in some cases, pulmonary edema,
- (2) An increase in plasma volume and a dilution of the serum proteins,
- (3) An increase in the heart rate, heart size, and cardiac output,
- (4) Gallop rhythm and systolic murmur,
- (5) An increase in oxygen consumption.

These phenomena and their relationship to congestive failure in humans are discussed. An explanation is offered for the stabilization of the peripheral venous pressure, which takes place during infusion.

The authors wish to thank Dr. C. Sidney Burwell for his helpful suggestions during the progress of these studies, and his aid in presenting their results.

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# THE EFFECT OF CALCIUM CARBONATE, ALUMINUM PHOSPHATE, AND ALUMINUM HYDROXIDE ON MINERAL EXCRETION IN MAN<sup>1</sup>

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## INTRODUCTION

In order to elucidate further the mechanism of the alkalosis observed during the alkali treatment of peptic ulcer, a detailed study of mineral excretion following the administration of relatively non-absorbable antacids seemed desirable. Hence, an investigation of the influence of calcium carbonate on mineral excretion in man was undertaken, and its action compared with the effects of aluminum hydroxide and aluminum phosphate, two non-absorbable compounds.

## METHOD OF STUDY

Studies were carried out in 2 adult men and one female with peptic ulcer, in all of whom previous observations had indicated renal function to be satisfactory. A special metabolic ward and nursing staff were utilized. The patients were maintained on special ulcer diets (Tables I and II), without change, during the entire experiment. Chloride-free distilled water was used for drinking purposes. The daily fluid intake averaged approximately

3000 cc. The daily urinary output varied from 1200 to 3000 cc. There was no significant change in body weight during the entire experiment. The only medication permitted, aside from the substances studied, was the occasional administration of cascara to facilitate daily bowel activity,<sup>2</sup> and the use of mild sedatives for sleep. An interval of 4 to 6 days was allowed for adjustment to the diet, prior to the control observation. Mineral excretion was studied over periods of 4 days duration each. Ten grains of carmine were given at the onset of each period and repeated 96 hours later; the feces obtained between the carmine markers was collected for analysis. The 4-day collection was mixed thoroughly; accurately weighed 2.0 gram samples were taken for the various determinations. All analyses were done in duplicate or triplicate.

The chemical studies of the feces were carried out as follows:

The calcium content was determined by first boiling the sample with concentrated nitric acid and potassium permanganate. After cooling, the mixture was diluted with distilled water to 100 (or 200) cc. volume and adjusted to the proper pH (4.6 to 5.2), using Brom Cresol green as indicator. The procedure then followed was the same employed for blood serum (1). (Calculation:  $\frac{\text{Titer}}{2} \times 10$  equals mgm. Ca per 1 gram feces.)

TABLE I  
Composition of diet employed in case A. K.  
(Daily values)

	Special diet	Standard values for sedentary adult female
Carbohydrates	198	
Protein	72	
Fat	104	
Calories	2016	
Chloride (mgm.)	2974	
Sodium (mgm.)	2029	
Potassium (mgm.)	3052	
Calcium (mgm.)	1159	800
Phosphate (mgm.)	1342	1320
Iron (mgm.)	10.5	12
Vitamin A (I.U.)	9570	5000
Thiamin (mgm.)	1.014	1.2
Riboflavin (mgm.)	2.51	1.8
Ascorbic Acid (mgm.)	78	70
Vitamin D (I.U.)	38	

TABLE II  
Composition of diet employed in cases  
C. D. and W. G.  
(Daily values)

	Special diet	Standard values 70 kgm. adult male
Carbohydrate	266	
Protein	58	
Fat	163	
Calories	2763	
Chloride (mgm.)	3047	
Sodium (mgm.)	2014	
Calcium (mgm.)	1140	800
Phosphate (mgm.)	1300	1320
Iron (mgm.)	7	12
Vitamin A (I.U.)	6200	5000
Thiamin (mgm.)	1.0	1.5
Riboflavin (mgm.)	1.2 to 2.4	2.2
Ascorbic acid (mgm.)	5 to 30	75
Vitamin D (I.U.)	160 to 385	

<sup>1</sup> This work was supported in part by a grant from John Wyeth and Bro., Inc., Philadelphia, Pa.

<sup>2</sup> There was no instance of diarrhea.



For determining the chloride content, 5.0 cc. of a 0.1 N solution of silver nitrate were added to 2.0 gram samples of feces and the mixtures allowed to stand for several hours. Digestion then was carried out as with the calcium analysis. The mixtures were cooled, 5 per cent ferric alum added as indicator, and then titrated with a 0.1 N solution of potassium thiocyanate. (Calculation:  $\frac{5 - \text{titer} \times 3.5}{2}$  equals mgm. chloride ion in 1.0 gram feces.)

The phosphate in the feces was determined in Case C. D. by wet ashing of the samples with nitric and sulphuric acids and, when necessary, superoxal. The mixtures then were cooled, adjusted to known volume with distilled water, and the phosphate content measured by the Fiske-Subbarow method (2) as adapted for the Evelyn photoelectric colorimeter. In Cases A. K. and W. G., the samples of feces were ashed in a muffle furnace and the analyses then carried out as above.

In Case A. K., the sodium content of the feces was measured by the following procedure: 20 cc. of ferric sulphate and 3 cc. of concentrated sulphuric acid were added to 10 gram samples. The mixtures were dried in an oven and then ashed in a muffle furnace. The ash was taken up in 10 cc. of distilled water and 3 cc. aliquots were analyzed for sodium by the method employed for blood (3).

The potassium content of the feces in Case A. K. was determined as follows: 2.0 gram samples of feces were dried in an oven and then ashed in a muffle furnace. The ash was taken up in 10 cc. of distilled water and 1.0 cc. aliquots were analyzed by the procedure used for blood (4).

Twenty-four hour collections of urine were obtained throughout the study. The urine was voided directly into a bottle containing toluene as a preservative and a layer of mineral oil to prevent the escape of  $\text{CO}_2$  (5). The urine was kept in an icebox and analyzed at the end of each collection for the following: (a) pH, using the Beckman pH meter, (b) chloride (6), (c) calcium, (d) phosphate, and (e) amino-nitrogen plus ammonium salts (7).<sup>8</sup>

The sodium content of the urine in Case A. K. was measured by the same technique employed for blood, modified as to the volume used; ferric salt was added in amounts sufficient to prevent interference by phosphates. The urinary potassium was determined as follows: 2.0 cc. samples of urine were ashed in a muffle furnace and the ash taken up in 10 cc. of distilled water; 2.0 cc. aliquots then were concentrated and analyzed by the method employed for blood.

Simultaneous studies were made of the serum electrolytes. Venous blood drawn under oil was utilized for the following analyses: chloride,  $\text{CO}_2$  (8), pH (9), calcium, and phosphorus. Oxalated blood was used for the measurement of the blood urea nitrogen (10). The following

<sup>8</sup> Referred to in the text as "ammonia" for the purpose of simplicity.

additional data were obtained in Case A. K.: cell volume, serum water (11), blood sodium, potassium, and total base (12), and the urea clearance (13).

After control values had been established, the effects of calcium carbonate, aluminum phosphate, and aluminum hydroxide were studied in individual periods. A 4 to 5 day interval for adjustment to the added medication was allowed prior to the analyses. Calcium carbonate was administered in 2.0 gram amounts 10 times daily: 80 grams (400 m.eq.) were thus given in a 4-day period, containing 32 grams of calcium ion. One hundred and five cc. of aluminum phosphate were administered daily in divided doses; a total of 420 cc. (35 m.eq.  $\text{HPO}_4$ ) in a 4-day period containing, by analysis, 386 mgm. of chloride, 4368 mgm. of phosphate, and 2625 mgm. of aluminum (14). One hundred and five cc. of aluminum hydroxide were given daily in divided doses; a total of 420 cc. in a 4-day period containing 386 mgm. of chloride and 4716 mgm. of aluminum. Complete mineral balances were not obtained since no attempt was made to measure the loss in the insensible perspiration or sweat. This loss presumably was constant, however, through the various periods in each case, since fairly constant conditions were maintained. The results are considered exclusively in relation to the individual control values.

## RESULTS

The complete data are recorded in Tables III and IV.

## DISCUSSION

### (1) Calcium carbonate

The ingestion of 400 m.eq. daily, as calcium carbonate, was roughly accounted for by increase in total outgo, almost entirely in the stools. The increase in excretion in the urine was, for the patient A. K., only 7.3 m.eq., and for the other 2 patients, C. D. and W. G., 13.9 m.eq. and 14.1 m.eq., respectively. As was to be expected with the large addition to calcium excretion in the stools (15), there was an increase of  $\text{HPO}_4$  in the feces. For A. K. and W. G., the outgo in the stools was about double the fore-period values. These increments were, however, somewhat more than offset by a decrease in the removal of  $\text{HPO}_4$  in the urine. The increase in calcium and decrease in  $\text{HPO}_4$  excretion in the urine together markedly reduced the requirement for ammonium production as noted in Table III. Other compounds of acid-base excretion, however, are related to the change in ammonium production. For the patient A. K., a small increase in Na and decrease in Cl, and a considerable reduction of K, were found. The re-

TABLE III  
Effect of calcium carbonate, aluminum phosphate, and aluminum hydroxide on mineral excretion in man

Subject	Periods of study (4-day)	Urine						Feces						Total output					
		pH	NH <sub>4</sub>	Na	K	Ca	HPO <sub>4</sub> *	Cl	Na	K	Ca	HPO <sub>4</sub> *	Cl	Na	K	Ca	HPO <sub>4</sub> *	Cl	
		m.eq. per 24 hours						m.eq. per 24 hours						m.eq. per 24 hours					
A. K. Unit No. 148736 Female Age 63 Gastric ulcer	Fore-period, I	6.16	34.3	54.5		11.6	31.2	72.8	1.3		30.6	10.5	2.0	55.8		42.2	41.7	74.8	
	Fore-period, II	6.27	35.6	59.0	63.3	12.6	30.5	82.3	0.9	6.6	41.3	10.9	2.0	59.9	69.9	53.8	41.4	84.3	
	Average	6.21	35.0	55.7	63.3	12.1	30.9	77.6	1.1	6.6	36.0	10.7	2.0	57.9	69.9	48.0	41.6	79.6	
	CaCO <sub>3</sub> , I (400 m.eq.)	6.56	20.6	58.8	49.1	20.5	12.1	71.0	1.7	11.9	356.0	24.9	4.1	60.5	61.0	376.5	37.0	75.1	
	CaCO <sub>3</sub> , II	6.75	21.0	69.3	52.1	20.2	10.4	81.2	1.9	12.1	394.0	24.8	3.9	71.2	61.2	414.2	35.2	85.1	
	CaCO <sub>3</sub> , III	6.63	26.2	49.5	50.2	17.5	12.7	73.2	0.7	12.0	378.0	20.5	4.0	50.2	62.2	395.5	33.2	77.2	
	Average	6.65	22.6	59.2	50.5	19.4	11.7	74.8	1.4	12.0	376.0	23.8	4.0	60.6	62.5	395.4	35.1	79.1	
	Change	+ 0.44	- 12.4	+ 3.5	- 12.8	+ 7.3	- 19.2	- 2.8	+ 0.3	+ 5.4	+ 340.0	+ 12.7	+ 2.0	+ 2.7	- 7.4	+ 347.4	- 6.5	- 0.5	
	After-period	5.92	36.6	70.5	60.6	8.6	30.4	85.2	2.0	10.6	38.8	9.7	2.7	72.5	71.2	47.4	40.1	87.9	
	Fore-period	5.76	67.5			12.0	26.7	66.6			54.3	14.4	2.5			66.3	41.4	69.1	
C. D. Unit No. 255381 Male Age 37 Duodenal ulcer	CaCO <sub>3</sub> (400 m.eq.)	6.24	37.2			25.9	8.9	23.77			484.0	17.8	2.9			509.9	16.7	26.6	
	Change	+ 0.48	- 30.4		+ 13.9		- 17.8	- 42.9			+ 429.7	+ 3.4	+ 0.4			+ 443.6	- 24.7	- 42.5	
	Aluminum phosphate, I (35 m.eq. HPO <sub>4</sub> )	5.42	88.4			9.5	24.5	87.1			46.3	41.1	4.3			55.8	65.6	91.4	
	Aluminum phosphate, II (35 m.eq. HPO <sub>4</sub> )	5.43	77.2			8.9	29.6	76.0			51.4	42.7	4.6			60.3	72.3	80.6	
	Average	5.42	82.8			9.2	27.1	81.6			48.9	41.9	4.5			58.1	69.1	86.0	
	Change	- 0.34	+ 15.3		- 2.8		+ 0.4	+ 17.0			- 5.4	+ 27.5	+ 2.0			- 8.2	+ 27.7	+ 16.9	
	Aluminum hydroxide	5.93	46.8			12.1	7.3	60.5			51.9	33.2	3.9			64.0	40.5	64.4	
	Change	+ 0.17	- 20.7			+ 0.1	- 20.7	- 6.1			- 2.4	+ 18.8	+ 1.4			- 2.3	- 0.9	- 4.7	

TABLE III—Continued

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TABLE III—Continued

Subject	Periods of study (4-day)	Urine										Feces					Total outgo						
		pH	NH <sub>4</sub>	Na	K	Ca	HPO <sub>4</sub> *	Cl	m.eq. per 24 hours					Na	K	Ca	HPO <sub>4</sub> *	Cl	m.eq. per 24 hours				
W. G. Unit No. 144032 Male Age 51 Gastric ulcer	Fore-period	5.16	73.0			6.3	24.5	75.0															
	CaCO <sub>3</sub> (400 m.eq.)	6.06	38.9			20.4	8.2	57.7															
	Change	+	34.1			14.1	16.3	17.3															
	Aluminum phosphate (35 m.eq.)	0.9																					
	Change	5.12	86.7			5.5	24.5	80.5															
	Aluminum hydroxide	0.04	13.7			0.8	0.0	5.5															
	Change	5.59	43.3			5.5	8.1	73.3															
		+	29.7			0.8	16.4	1.7															

\* HPO<sub>4</sub> is here taken as univalent for the reason that at the usual pH of urine its base equivalence is approximately 1.0. The values for "change" are derived by comparison with the fore-period values.

\* HPO<sub>4</sub> is here taken as univalent for the reason that at the usual pH of urine its base equivalence is approximately 1.0.  
The values for "change" are derived by comparison with the fore-period values.

TABLE IV

*Effect of calcium carbonate, aluminum phosphate, and aluminum hydroxide on various constituents of the blood plasma*

Subject	Periods of study (4-day)	Cl	CO <sub>2</sub>	pH	P	Ca	K	Na	Total base	BuN	Urea clearance	Cell volume	Scrum water
		mM./L.			mM./L.		m. eq./L.			mgm. per cent	per cent average normal	per cent	grams per cent
A. K. Unit No. 148736 Female Age 63 Gastric ulcer	Fore-period I	101.9	30.9	7.43	1.26	4.9	5.30	143.0	155.3	15.4	66	40.4	91.1
	Fore-period II	102.2	31.8	7.47	1.29	4.7	5.20	144.3	153.3	14.6	81	37.0	90.97
	Calcium carbonate I	100.7	31.7	7.50	1.35	5.75	5.20	147.9	160.0	17.0	44	41.0	90.42
	Calcium carbonate II	100.5	31.9	7.44	1.19	5.35	5.00	145.2	158.5	17.8	65	41.5	90.68
	Calcium carbonate III	103.1	29.7	7.47	1.22	5.1	5.30	145.0	156.1	15.5	65	37.5	91.14
	After-period	100.2	30.9	7.43	1.13	5.1	5.30	143.8		13.2	76	37.2	91.06
C. D. Unit No. 255381 Male Age 37 Duodenal ulcer	Fore-period	101.7	29.6	7.46	1.32	5.05				9.4			
	Calcium carbonate	99.6	29.6	7.46	0.97	5.15				13.5			
	Aluminum phosphate I	103.4	28.4	7.48	1.0	5.7				12.0			
	Aluminum phosphate II	100.5	29.8	7.46	1.22	5.5				10.0			
	Aluminum hydroxide	102.4	28.5	7.47	1.13	5.25				15.4			
W. G. Unit No. 144032 Male Age 51 Gastric ulcer	Fore-period	96.7	28.6	7.43	1.42	4.65				14.2			
	Calcium carbonate	95.6	29.9	7.43	1.35	5.1				13.8			
	Aluminum phosphate	106.9	28.2	7.42	1.40	4.75				16.3			
	Aluminum hydroxide	101.6	27.4	7.41	1.40	5.05				14.0			

maining components, Mg, SO<sub>4</sub>, and organic acids were not measured. The accuracy of ammonium adjustment is shown by the slight extent of change in urine pH. For the patient W. G., a considerable, and for C. D., a much larger, reduction of chloride in the urine was found. Excretion of this anion in the urine is known to fluctuate widely even in the presence of a constant intake (16, 17).

The changes in mineral excretion induced by calcium carbonate occurred within 24 hours after the addition of the alkali to the regimen. Mineral excretion returned to original levels almost equally soon after the discontinuation of calcium carbonate therapy.

The use of calcium carbonate did not alter the electrolyte constitution of the blood plasma (Table IV).

### (2) Aluminum phosphate

The ingestion of 35 m.eq. HPO<sub>4</sub> as aluminum phosphate caused a roughly equivalent increase in the stools and had no appreciable effect on HPO<sub>4</sub> output in the urine. Calcium excretion in the stools was to a slight extent reduced. There was no appreciable change in the calcium output in the urine. Some increase in chloride and, also, ammonium excretion in the urine was found. A relationship of these changes to phosphate ingestion was not apparent. No alterations were noted in the electrolyte components of the blood plasma.

### (3) Aluminum hydroxide

Aluminum hydroxide caused a considerable increase in HPO<sub>4</sub> excretion in the stools, as demon-

strated by the data from the two periods of study. This finding is in agreement with the results obtained by other workers (18). A roughly equivalent decrease of the excretion of  $\text{HPO}_4$  in the urine was found and, along with it, the to be expected reduction of ammonium. For patient C. D., this was exactly equivalent to the decrease of  $\text{HPO}_4$ . The total outgo of  $\text{HPO}_4$  was not increased. Aluminum hydroxide did not alter the electrolytes of the blood plasma, thus confirming previous observations (19).

### CONCLUSION

The ingestion of calcium carbonate, aluminum phosphate, or aluminum hydroxide, in the quantities used in the treatment of peptic ulcer, places no appreciable burden on the processes of acid-base metabolism. The necessary adjustments of acid-base excretion are relatively small and are accomplished with a remarkable precision.

The electrolyte constitution of the blood plasma is not disturbed.

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# THE EFFECTIVE STIMULUS FOR INCREASED PULMONARY VENTILATION DURING MUSCULAR EXERTION

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The control of the pulmonary ventilation is a central problem in the physiology of muscular exercise. The hypothesis that the respiratory center is stimulated by chemical changes in the arterial blood has been defended by Gesell (1), Haldane (2), and Henderson (3). In addition to this type of stimulation, Krogh and Lindhard (4) have discussed the anticipatory hyperventilation preceding and immediately following the onset of exercise. This they attribute to cerebral cortical stimulation. Recently, Harrison and his associates (5, 6) have maintained that the stimulus for the increase in pulmonary ventilation in men and dogs during very moderate exercise, either active or passive, is not chemical, but is reflex. Comroe and Schmidt (7) confirmed these findings in anesthetized dogs, using either electrical stimulation of the lumbar anterior spiral roots, or else passive movements of the limbs. In view of the importance of this problem, we have repeated Harrison's experiments, which use light exercise, and have extended them to include a more strenuous grade of exercise, in which the changes in ventilation are larger and more consistent from experiment to experiment and from subject to subject.

## EXPERIMENTAL METHODS

Two different types of experiments have been made:

(1) In the first type, using the arm muscles of recumbent subjects, ventilation was measured: (a) at rest; (b) at rest, with the circulation of both arms cut off at the level of the distal insertion of the deltoids; (c) during exercise of the flexors of the hand, with the circulation normal; (d) during the same exercise continued to exhaustion, with the circulation cut off; (e) immediately after restoration of the circulation. After each period of exercise the subject was allowed to rest until he regained his basal condition. The exercise consisted in squeezing inflated rubber bulbs with each hand 60 times per minute at a rate of work of 12 kilogrammeters per minute for 1½ minutes, or squeezing a bulb with one hand and moving an ergograph with the other. Sphyg-

momonometer cuffs inflated to 200 mm. Hg were used to cut off the circulation.

(2) In the second type, the leg muscles were used. The subject walked on a motor-driven treadmill, at a grade of 8.6 per cent, and a speed of 2.4 or 3.5 m.p.h., until a steady state was reached. While still walking, the circulation of both legs was cut off by inflating, to a pressure of 200 mm. Hg, sphygmomanometer cuffs placed around the legs at the level of the distal insertion of the gluteal muscles. After a given time, usually 1½ minutes, the pressure was relieved and the subject continued to walk, until the previous steady state was reached, usually within 10 minutes. Ventilation was measured during this steady state, then during the ischemia, and finally after release of the pressure.

## RESULTS

Table I shows the results of the first type of experiments, in which recumbent subjects exercise

TABLE I  
*Pulmonary ventilation of recumbent normal subjects under conditions stipulated*

Subject	Experiment number	Condition of subject				
		Resting	Resting and ischemic	Exercising, no ischemia	Exercising, arms ischemic	Resting, first minute after ischemia
		<i>liters per minute, dry at S.T.P.</i>				
Bar	1	6.90	7.00	8.10	7.30	12.4
	2	7.50	8.20	8.90	8.90	
	3	7.20	8.00	8.50	8.50	13.0
	4	6.40	7.60	7.60	7.00	12.2
	5	6.80	7.80	8.10	7.80	14.5
	6	6.80	7.50	9.00	8.10	
	7	6.70	6.90	6.80	7.10	13.5
	8	6.70	6.70	7.30	7.30	12.5
	9	6.72	7.20	7.20	7.20	16.0
	10	7.00	7.80	7.20	7.00	
Mor	1	6.70	9.00	8.90	8.90	12.3
	2	7.74	7.74	8.40	7.90	12.3
	3	7.80	8.20	7.90	9.10	13.1
	4	6.80	7.60	7.70	7.80	
	5	6.80	6.90	8.60	9.10	
	6	7.95	8.20	8.45	8.00	
	7	6.75	6.80	7.43	6.75	
	8	7.80	7.85	7.85	7.20	12.8
	9	8.10	8.00	9.90	9.00	
	10	7.80	8.00	9.50	10.00	15.0
	11	6.90	8.10	9.10	8.00	
Hol	1	6.80	8.10	8.50	8.20	14.0
	2	6.50	7.10	8.80	8.20	12.0

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the flexors of the hands. It should be compared with Table III of Harrison (5), and differs from his results in the following ways: (1) mere ischemia nearly always produced a slight increase in ventilation at rest; (2) exercising the flexors of the hands, without ischemia of the arms, always increased the ventilation, but to a far smaller extent than shown by Harrison; (3) exercising the flexors of the hands with the arms ischemic usually produced a decrease in ventilation below the value for the same exercise with circulation intact; (4) release of the ischemia produced at once a marked increase in ventilation (this increase was not remarked upon by Harrison (5), nor is it shown in his tables); (5) the changes were so small that their significance is hard to evaluate, except in the case of the marked hyperventilation after ischemia.

Our results, therefore, lead to the following conclusions: (1) that ischemia during exercise of this very mild sort usually results in diminution in ventilation in comparison with exercise with normal circulation; and, (2) that after ischemia is released, a large increase in ventilation occurs. These findings can only be explained by chemical stimulation of the respiratory centers and not by reflex stimulation. In these experiments, the external work was about 12 kilogrammeters per minute, and the extra oxygen consumption of the subjects was only about 30 cc. per minute, a figure which agrees well with the calculated work done.

In view of the small changes in ventilation produced by this type of exercise, and the difficulty

in standardizing the work, we decided to employ a well-standardized exercise, easy to control, and producing relatively large changes in ventilation. In this way, we hoped to get consistent, clear cut results.

Table II shows typical results of experiments with subjects walking on the treadmill, first in a steady state, then with ischemia of both legs, and finally after the release of the ischemia (*cf.* experiments of type 2 in Experimental Methods). The data for ventilation in the first one or two experiments on each subject show some variability. However, after the subjects became used to the discomfort of the experimental condition, ischemia always produced a marked decrease in pulmonary ventilation, to be followed after release of the ischemia by a marked rise above the level of the steady state and a return, within 10 minutes, to the level of the steady state. It will be seen that ischemia in every experiment produced a marked decrease in the oxygen consumption, and release of the pressure was always followed by a marked increase above the level of the steady state.

The data in Table II lead to the following conclusions and interpretations:

(1) Subjects must be exposed to this type of experiment several times before the results become reliable.

(2) Even though the nervous connections remain intact, total ischemia of the legs during walking causes a decrease in the pulmonary ventilation.

TABLE II

*Pulmonary ventilation and oxygen consumption in normal men walking at a speed of 3.5 m.p.h. and a grade of 8.6 per cent.*

Subject	Condition	Pulmonary ventilation				Oxygen consumption			
		1st expt.	2nd expt.	3rd expt.	4th expt.	1st expt.	2nd expt.	3rd expt.	4th expt.
		<i>liters per minute</i>				<i>liters per minute</i>			
Bar	Steady state, no ischemia	46.3	43.0	43.7	43.7	1.485	1.570	1.500	1.525
	During ischemia	43.4	43.9	36.9	38.2	1.115	1.200	1.142	1.146
	First minute after ischemia	64.0	54.5	65.4	55.4	1.860	2.000	2.000	1.950
	Ten minutes after ischemia	48.0	44.5	44.2	44.0	1.500	1.590	1.580	1.560
Mor	Steady state, no ischemia	29.6	34.0	36.7	36.0	1.320	1.400	1.360	1.390
	During ischemia	28.0	39.2	29.9	33.5	1.000	1.090	1.100	1.080
	First minute after ischemia	43.2	54.3	46.3	50.1	2.210	2.000	2.100	2.050
	Ten minutes after ischemia	32.1	33.6	38.8	35.2	1.320	1.420	1.400	1.410
Con	Steady state, no ischemia	48.6	48.0	45.0		2.490	2.400	2.480	
	During ischemia	45.4	43.0	43.2		1.560	1.610	1.680	
	First minute after ischemia	70.5	70.0	68.8		3.425	3.510	3.240	
	Ten minutes after ischemia	48.2	50.0	47.0		2.480	2.390	2.490	

The stimulus to ventilation during walking must therefore be chiefly of chemical, not reflex, origin.

(3) After release of the ischemia, even though the nervous connections remain unaffected, there is a marked increase of ventilation which must be caused by chemical stimuli. This conclusion has been verified by estimation of the blood lactate, and the results will be discussed elsewhere (8).

#### DISCUSSION

Our results are in agreement with the hypotheses of Gesell (1), Haldane (2), and Henderson (3) that the respiratory centers are stimulated chemically, whether it be by the pH of the arterial blood, or by other circulating factors affecting the intrinsic metabolism of the centers, and with the findings of Jarisch and Gaisböck (9) with resting men, in whom ischemia was accomplished by a significant decrease in oxygen consumption, and in whom, relief of the ischemia produced at once a hyperventilation with increased oxygen consumption.

The discrepancies between our results, using very light exercise, and Harrison's (5) are difficult to explain. It is possible that the anticipatory hyperventilation shown by Krogh and Lindhard (4) plays some part in Harrison's results. We have found that even subjects well accustomed to the procedures involved in studying muscular exercise in the laboratory, showed variable results during the first few experiments, and only after several days began to give consistent results. In our experience, no normal subject accustomed to the experimental procedure has had a ventilation as high as those reported by Harrison (5), under experimental conditions similar to his. The chief difficulties with using light exercise are that the work is hard to standardize, and that the changes in ventilation are small. When a heavier grade of work is used, these difficulties disappear, since voluntary and subconscious factors which can easily influence pulmonary ventilation in man at rest or doing very light exercise, become less and less effective with increasing rates of muscular work. In fact, during exercise that exhausts within a few minutes, voluntary alteration of the pulmonary minute-volume is virtually impossible.

All of the results reported in the present paper are consistent with the hypothesis that increase in ventilation in exercise is caused predominantly by

chemical stimulation, and not by reflex stimulation from the leg.

#### SUMMARY

1. The increase in pulmonary ventilation has been studied in normal men exercising the limbs, first with normal circulation, and then with the circulation cut off by pressure from inflated cuffs.

2. Very light exercise (at a rate of 12 kilogrammeters per minute) of the flexors of the hand during total ischemia of the arms usually resulted in a smaller ventilation than when the arms had normal circulation. Release of the ischemia was followed at once by a marked hyperventilation.

3. In subjects walking uphill on a treadmill (at a rate of 700 kilogrammeters per minute for a 90 kilo subject), total ischemia of both legs resulted in a marked diminution of pulmonary ventilation, even though the nervous pathways were intact, and release of the ischemia was followed at once by a marked hyperventilation, much higher than the value prior to ischemia.

4. These results are interpreted to mean that the chemical stimulus for increased ventilation in exercise of these types is far more important than the reflex.

5. The discrepancies between our results and Harrison's (5) are perhaps due to the fact that consistent results are found only after the subjects have become well accustomed to the experimental procedures by several repetitions of the experiments, and when exercise of sufficient severity is used so that the changes in ventilation and oxygen consumption are relatively large in magnitude.

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# ATYPICAL PNEUMONIA AND PSITTACOSIS

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Attention has been focused in recent years on the acute infectious pulmonary disease which, for want of a better name, has been called "atypical pneumonia." Many descriptions of this type of malady have appeared (1 to 10), and several different non-bacterial infectious agents have been incriminated or identified as the cause of certain of the outbreaks (3, 10 to 14). These findings indicate that atypical pneumonia is a clinical-pathological entity which has a diverse etiology. One of the first approaches to the problem of atypical pneumonia should be the establishment of an etiological diagnosis whenever possible. In this way, certain types of cases can be removed from the general group and studied in a more intelligent manner.

The fact that infection with psittacosis virus is responsible for certain cases of non-bacterial pneumonia is well recognized. For example, pulmonary consolidation is a characteristic feature of classical psittacosis in man, caused by psittacine strains of the virus (15). Furthermore, Eaton and his co-workers (13) have demonstrated that a strain of psittacosis virus, which is practically indistinguishable (13, 16 to 18) from the agent designated meningopneumonitis by Francis and Magill (19), can cause pneumonia and death in human beings. Finally, Meyer and his associates (20 to 22) have emphasized the role of other members of the psittacosis group in producing bronchopneumonia; recently, they have laid stress on the transmission of certain strains of the virus to man from infected pigeons and chickens.

It is appropriate to discuss at once the confusion that exists regarding the exact relationship of a number of viruses, which have been isolated by several groups of workers from different sources, and which have many characteristics in common with one another and with classical psittacine strains of the virus of psittacosis. Thus, Rake, Eaton and Shaffer (16) have demonstrated that sera of patients convalescent from lymphogranuloma venereum, from psittacosis of parrot or

pigeon origin, and from the pneumonitis studied by Eaton, all possess an antibody which fixes complement in the presence of antigen prepared from the virus of lymphogranuloma venereum, of psittacosis, or of meningopneumonitis. Furthermore, these workers, as well as Pinkerton and Moragues (17), have noted similarities in the morphological and tinctorial qualities of these viruses, and in the character of the lesions produced by them in experimental animals. Although certain differences in the morphological and antigenic properties of the viruses and in the disease they produce in experimental animals have suggested that they are not identical (16 to 18, 23, 24), these observed differences are less impressive than the similarities. Because of the obviously close relationship of the viruses and because of their tendency to produce latent infections with the persistence of immunological phenomena, rigid diagnostic criteria must be fulfilled before one can state that a given acute infection is caused by a member of this group of agents.

This report concerns itself primarily with the incidence of infections caused by strains of the psittacosis virus among patients with atypical pneumonia, who were encountered in a random sampling of the disease in large eastern urban areas.

## MATERIALS AND METHODS

*Serological studies.* Samples of patients' sera were studied for antibodies against the viruses of psittacosis, lymphocytic choriomeningitis, influenza A, and influenza B. Titrations of influenza antibodies were made by Dr. F. L. Horsfall, Jr., of the Rockefeller Hospital as part of a coöperative investigation on the etiology of atypical pneumonia.

*Psittacosis.* Psittacosis antigen for complement fixation titrations with patients' serum was prepared from agar slant culture material (25), infected with the 6 BC strain of virus isolated from a parakeet by Dr. K. F. Meyer in 1941. Our methods for the preparation of suspensions of washed inactive virus and our technique of complement fixation are modifications of those recommended by Meyer and Yanamura (26). Antigen is prepared as follows: Infected tissue is removed from the agar surface of one

Kolle flask, which had been inoculated 5 days previously with minced chicken embryo tissue and culture virus, and triturated with 50 cc. of buffered water, *i.e.*, Sørensen's phosphate buffer, pH 7.6, diluted 1:50. The tissue suspension is centrifuged in 25 cc. amounts in 50 cc. centrifuge tubes, at 3000 r.p.m., for 10 minutes in the International machine. The supernatant fluid is saved for high speed centrifugation and the sediment is thoroughly resuspended in 40 cc. of buffered water and recentrifuged; again the supernatant fluid is saved. The fluid obtained from the first centrifugation is placed in capped lusteroid tubes and spun at 12,400 r.p.m., in the angle centrifuge of Pickels (27), for 30 minutes in the cold room. The supernatant fluid is discarded and the sediment material is resuspended, by means of a capillary pipette, in the fluid saved from the second preliminary run in the International machine. This suspension is again spun at 12,400 r.p.m. for 30 minutes, and the resultant sediment is resuspended in 50 cc. of physiological saline solution, buffered at pH 7.6. This preparation of washed virus is freed of large aggregates by a final centrifugation at 2000 r.p.m. for 10 minutes. A portion of the material is saved for a titration of its infectivity; such material, when inoculated into mice by the intracerebral route, generally has a titration endpoint of  $10^{-7}$  to  $10^{-8}$ . The suspension of virus is rendered non-infectious by heating in a steam bath at 100 C. for 30 minutes. Only non-infectious antigens are used in the complement-fixation tests.

The complement-fixation test is performed as follows: 0.2 cc. amounts of antigen, 2 units, are mixed with 0.2 cc. volumes of serial two-fold dilutions, beginning at 1:8, of the serum to be tested, and then 0.5 cc. of diluted complement, 2 units titrated in the presence of antigen, are added. After the mixture has been incubated for  $2\frac{1}{2}$  hours at 37 C., the hemolytic system is added; this consists of 0.2 cc. of hemolysin appropriately diluted to contain 2 units and 0.5 cc. of a 2 per cent suspension of washed sheep erythrocytes. The test materials are incubated at 37° C. for one-half hour when final readings are made. Titration end points are estimated on the basis of the last tube showing complete or three plus fixation, and the titer represents the dilution of serum originally added to that tube. In addition to the controls usually employed in complement fixation tests, each serum is titrated against a control antigen which is prepared in exactly the same way as the test antigen, except that the culture flask is not infected with virus.

*Lymphocytic choriomeningitis.* Soluble antigen of lymphocytic choriomeningitis was employed in the complement fixation tests for antibodies against this virus infection. Instead of our usual technique (28) for demonstrating fixation with choriomeningitis materials, a modification of the procedure described for the psittacosis test was adopted in order to simplify the work. The two methods gave equally satisfactory results with choriomeningitis, but the former was less desirable for psittacosis. Samples of serum from each patient were titrated for antibodies against the soluble antigen of choriomeningitis over the range of dilutions from 1:2 to 1:32. Pre-

vious work had demonstrated that these immune substances are detectable in human sera during only a few weeks in convalescence, *i.e.*, usually between the third and sixth weeks (28). Therefore, results were recorded as negative only for those patients whose sera taken during this period failed to fix complement in the presence of soluble antigen.

*Influenza A and B.* Samples of serum were titrated for antibodies against influenza A and B by means of the agglutination-inhibition technique of Hirst (29). Serial two-fold dilutions of the serum to be tested were prepared, and to 0.5 cc. amounts of each dilution were added 0.5 cc. volumes of suspensions of A and B virus, obtained from allantoic fluid of infected eggs; the PR-8 and Lee strains, respectively, were employed. Suspensions of virus for the test were known to contain 4 units of active material, *i.e.*, 4 times the concentration which caused clumping of 50 per cent of the chicken erythrocytes in a test suspension (29). One cc. amounts of a 1.5 per cent suspension of washed chicken red cells were then added to the serum-virus mixtures, and readings were made after the tests had stood for an hour at room temperature. The titer of antibody was taken as the highest dilution of serum which was capable of inhibiting the agglutination reaction sufficiently to prevent the aggregation of half the red cells.

The diagnostic importance of demonstrating a rise in titer of antibodies of influenza viruses in the sera of patients during convalescence has been emphasized (30); therefore, whenever possible, a sample of serum taken during the first few days of illness and one taken later were tested. When this was not feasible, the antibody levels in single convalescent sera were determined. While too great diagnostic significance cannot be attached to the results obtained with a single serum, certain ranges of antibody levels have been reported for apparently normal individuals (31). Therefore, one can say that the titers are within the normal limits and that the patient probably had not had a recent infection, or that the antibody level for one of the viruses is distinctly elevated and that the patient may have had contact with this virus within the last few months.

*Isolation of psittacosis virus.* Isolation of virus from human material was attempted in only a few instances; this was due in part to the scarcity of proper specimens. For example, only one of the 2 patients who contracted psittacosis from infected pigeons of the G. flock coughed up sputum and this individual produced only 2 specimens; yet both patients presented x-ray evidence of extensive pulmonary consolidation. Specimens for virus studies, *i.e.*, sputum, throat washings, and lung tissue, were stored at -70 C., generally until the results of serological tests were available. Only materials from the patients whom we suspected of having psittacosis were examined in our laboratory. Specimens from patients with atypical pneumonia unassociated with psittacosis were investigated by Dr. F. L. Horsfall, Jr.

A study of the characteristics of the strains of virus of the psittacosis group which were isolated from the 2

patients A. G. and B. T., and from the pigeons of the G. flock, is in progress. It need only be stated at this time that strains isolated from these sources have many properties in common with the strains of psittacosis virus isolated from psittacine birds (32) and from pigeons (21), and with the virus of meningopneumonitis (16).

## RESULTS

Previous experimental studies on the etiology of atypical pneumonia have generally dealt with small outbreaks of the disease (10 to 14) or with individual cases in whom a history of contact with sick birds suggested the possibility of psittacosis (22). A somewhat different approach to the problem was made in the present investigation. Cases of atypical pneumonia sporadically distributed in several large eastern cities were selected at random and investigated with the object of determining the incidence of infection with certain of the viruses which might be expected to cause pulmonary involvement. We were interested primarily in disease produced by members of the psittacosis group of viruses, but also investigated the possible role of the viruses of lymphocytic choriomeningitis and of influenza A and B as etiological agents of the atypical pneumonia present in our patients. We have been impressed by the high incidence of psittacosis in such patients and by the frequency with which some sort of association can be demonstrated between the patients and non-psittacine birds, infected with one of the psittacosis group of viruses.

### *Atypical pneumonia caused by the virus of psittacosis*

The clinical picture observed in patients with psittacosis infection varied considerably; moreover, no distinguishing features were encountered in this group which permitted a clean cut separation of these patients from the group of individuals who had atypical pneumonia due to some other cause. In a like manner, histopathological examination of human lung tissue failed to reveal significant differences between the pulmonary lesions seen in the one specimen from which psittacosis virus was isolated and in the two specimens from which the virus was not obtained. The lesions in all three instances were like those described by McCordock and Muckenfuss as characteristic of the response of lung tissue to infection

with a number of viruses (33). In brief, the clinical and pathological findings in our group of sporadic cases of atypical pneumonia of known and unknown etiology were similar. Furthermore, our patients resembled in most respects those observed by Kneeland and Smetana (6) and by Longcope (7).

Emphasis has already been laid on the difficulties encountered in establishing proof of recent infection with a virus of the psittacosis group. Formerly, the demonstration, in a single sample of convalescent serum, of complement fixing antibodies which reacted with psittacosis antigen was considered adequate for diagnosis. Evidence of this nature is no longer conclusive in view of the ubiquity of agents capable of eliciting such antibodies, and of the long interval of time that these antibodies may persist. Therefore, certain minimal criteria have been selected arbitrarily as essential for the proof that a given illness is caused by a strain of psittacosis virus. These are (1) isolation of virus during the acute phase of the disease, or (2) the demonstration of the appearance of complement fixing antibodies during convalescence or of a significant rise in titer of these antibodies during the period of recovery. In other words, it is now imperative to adopt the same type of criteria for the diagnosis of psittacosis that previously has been found necessary for lymphocytic choriomeningitis (34) and influenza (30).

Technical difficulties involved in work with undiluted human sera in the test used for psittacosis have now led us to begin our titrations for psittacosis antibodies with a 1:8 dilution of serum. For a given specimen to be considered positive in our laboratory, it must give complete fixation in a dilution of serum of 1:16 or greater. Significance is attached to a rise in antibody titer during convalescence only if the increase is fourfold or more. Furthermore, for a mere fourfold increase to be considered valid, the early and late samples of serum must be tested in the same experiment.

Ten persons, among the 45 from eastern cities who had atypical pneumonia, suffered from psittacosis. Stated another way, on the basis of the criteria enumerated in the preceding paragraph, infection with a strain of psittacosis virus was proved to have been associated with the illness of almost one-fourth of the members of the group of

TABLE 1  
*Psittacosis among 45 cases of atypical pneumonia occurring in eastern cities.*

TABLE I Psittacosis among 45 cases of atypical pneumonia occurring in eastern cities.																
Laboratory diagnostic procedures																
Complement-fixation test																
Etiological diagnosis	Patient	Age	Color	Sex	Contact with sick birds	Elevated titers for influenza		Lympho-cytic chorio-meningitis	Early serum		Intermediate serum		Late serum		Isolation of virus	
						A	B		Titer	Week	Titer	Week	Titer	Week		
Psittacosis 10 patients	F.C.	years 50	W	F	None known.	No	No	Negative	1 : 32	2	1 : 8	4	1 : 128	9	Not attempted	
	A.G.	51	W	M	Repeatedly handled sick pigeons from G. flock. Virus of psittacosis group isolated from these pigeons.	No	No	Negative	1 : 32	2	1 : 64	3	1 : 256	5	Virus of psittacosis group from sputum	
	E.Gr.	52	W	F	Handled sick pigeons of G. flock.	No	No	Negative	Negative	1	1 : 128	5	1 : 128	6	Not attempted	
	E.Gu.†	52	W	F	Lives next door to pigeon flock L. No sick birds but 6 of 8 pigeons had C-F antibodies for psittacosis antigen.	No	No	Negative	1 : 8	2	1 : 64	4	1 : 64	5	Not attempted	
	R.L.†	53	B	M	Visits pigeon loft of friend. None of birds available for study.	No	No	Negative	Negative	1	1 : 32	2	1 : 64	5	Not attempted	
	J.M.†	23	W	M	Repeatedly handled pigeons of M. flock. No sick birds, but 2 of 6 birds had C-F antibodies for psittacosis antigen.	No	No	Negative	1 : 64	2	1 : 16	3	1 : 128	5	Not attempted	
	C.P.†	65	W	F	None known.	No	No	Negative	1 : 64	2	1 : 256	4	1 : 128	10	Not attempted	
	F.S.	62	W	F	Owning 2 canaries. One lost, other sent to Dr. K. F. Meyer who failed to demonstrate virus or C-F antibodies. Visited bird store downstairs for canary food.	No	No	Negative			1 : 64 post mortem 16th day				Virus of psittacosis group from lung tissue	
	B.T.†	44	W	F	Owned 2 canaries. One lost, other sent to Dr. K. F. Meyer who failed to demonstrate virus or C-F antibodies. Visited bird store downstairs for canary food.	No	No	Negative							Not attempted	
	J.W.†	52	W	M	Pigeon flock S. on roof of patient's house. 2 of 6 birds had antibodies for psittacosis antigen.	No	No	Negative	1 : 8	1	1 : 128	4				Not attempted
Possibly psittacosis 5 patients	C.M.	48	W	F	Indefinite. Sister and friends have canaries. None sick (?).	No	No	Negative	1 : 64	1	{ 1 : 128 1 : 64	3 4	partial 1 : 4	9	Not attempted	
	R.McK.	15	W	M	None known.	No	No	Negative	partial 1 : 8	2	1 : 16	4	1 : 64	9	Not attempted	
	C.O'D.	45	W	F	None known.	No	No	Negative	1 : 128	2	1 : 128	3	1 : 128	7	Not attempted	
	E.S.	60	W	F	None known.	No	No	Negative			1 : 64	3	1 : 64	5	Not attempted	
	M.T.	59	W	F	None known.	No	No	Negative			1 : 64	3	1 : 64	5	Not attempted	

† Drs. S. Frant, A. C. Eder, and M. Greenberg of the New York City Department of Health provided valuable assistance in epidemiological investigations on these patients.

TABLE 1—Continued.

Laboratory diagnostic procedures																
Etiological diagnosis	Patient	Age <small>years</small>	Color	Sex	Contact with sick birds	Other contacts	Complement-fixation tests									
							Elevated titers for influenza		Lympho- cytic meningitis	Psittacosis						
							A	B		Early serum		Intermediate serum		Late serum		
											Titer	Week	Titer	Week	Titer	Week
Not psittacosis (25 patients)	H.A.	24	W	M	None known.	Patient H.A.	No	No	Negative	2	1:4	4	1:2	6		
	M.B.*	19	W	M	None known.		No	Yes	Negative	2	Negative	3	Negative	10		
	A.B.	20	W	M	None known.		No	No	Not done	Negative	1	Negative	4	Negative	7	
	R.B.	9	W	M	None known.		No	No	Negative	2	Negative	4	Negative	6		
	D.C.	4	W	M	None known.		No	No	Negative	2	Negative	3	Negative	8		
	L.C.	28	W	M	None known.	Patient H.A.	No	No	Negative	1	1:4	5	Negative	14		
	J.D.	53	W	M	None known.		No	No	Negative	1	Negative	3	Negative	7		
	G.E.	33	W	F	Canary—not sick.		No	No	Negative	1	Negative	3	Negative	6		
	M.F.	29	W	M	None known.		No	No	Negative	1	1:2	1	1:2	9		
	W.G.	18	W	M	None known.		No	No	Negative	1	Negative	1	Negative	6		
	E.G.	27	W	F	None known.	Patient H.A.	No	No	Negative	1	Negative	4	1:2	5		
	A.H.	54	W	M	None known.		No	No	Negative	1	Negative	3	Negative	5		
	A.H.	46	W	M	None known.		No	No	Negative	1	Negative	4	Negative	6		
	T.L.	18	W	M	None known.		Significant decrease	No	No	Negative	1	Negative	3	Negative	6	
	E.L.G.	45	W	F	None known.		No	No	Negative	2	Negative	4	Negative	5		
	J.L.	30	W	M	None known.	Patient H.A. Died 4th week Died 3rd week	No	No	Negative	1	Negative	3	Negative	9		
	M.M.	25	W	F	Close contact with pigeons, none sick.		No	No	Negative	1	Negative	4	Negative	7		
	E.O.	43	W	F	None known.		No	No	Negative	1	Negative	4	Negative	9		
	R.S.	49	W	M	None known.		No	No	Negative	2	Negative	3	Negative	9		
	C.S.	37	W	M	None known.		No	No	Negative	2	Negative	3	Negative	9		
	D.S.	24	W	M	None known.		No	No	Negative	1	Negative	3	Negative	9		
	H.G.†	43	W	M	None known.		Not done	Not done	Not done	2	1:4	4	1:8	10		
	J.D.†	48	W	M	None known.		Not done	Not done	Not done	2	1:4	4	1:8	10		
	C.R.	53	W	F	None known.		No	No	Negative	2	1:8	2	1:8	10		
	B.S.	13	W	F	None known.		No	No	Not done	2	1:4	2	1:4	10		
Probably not psittacosis 5 patients)	A.B.	19	W	M	None known.	Probably not psittacosis 5 patients)	No	No	Negative	2	1:4	4	1:8	10		
	C.B.	41	W	M	None known.		No	No	Negative	2	Negative	4	Negative	10		
	E.D.	48	W	F	None known.		No	No	Negative	2	Negative	4	Negative	10		
	M.M.	41	W	F	None known.		No	No	Negative	2	Negative	4	Negative	10		
	S.P.	6	W	F	None known.		No	No	Not done	2	1:4	2	1:4	10		

\* Psittacosis virus not obtained.

\* Psittacosis virus not obtained from sputum and throat washings.

† Psittacosis virus not obtained from lung tissue.

sporadic cases of atypical pneumonia. A virus of the psittacosis family was isolated from sputum of one patient and from lung tissue of another. The diagnosis of psittacosis which was made on the remaining 7 persons depended entirely on results of serological studies. A summary of pertinent data on the 10 cases of proved psittacosis is included in Table I, together with information on the findings in the remaining 35 members of the group with atypical pneumonia.

Psittacosis virus may have been of etiological importance in 5 additional patients with atypical pneumonia but this remains unproved. Only the results of serological investigations implicate the virus in the disease of these 5 individuals, and, although each person had significant amounts of complement fixing antibodies in his convalescent serum, none developed a fourfold increase in titer of these substances during the period of study. It is not unlikely that we have been too drastic in our choice of minimal standards for the diagnosis of psittacosis infections in human beings, and that some or all of these 5 patients should be included as cases of psittacosis. Indeed, Meyer (20) did not hesitate to make the diagnosis of psittacosis in 2 women (O. W. and E. W., patients of Dr. George S. Mirick of the Rockefeller Hospital) on the basis of his serological studies, although the evidence in these 2 instances was no more impressive than that obtained on C. M. and F. S. (Table I). Nevertheless, until increased experience is gained in this field where serological reactions are so involved, it seems preferable to maintain a conservative viewpoint.

#### *Atypical pneumonia unassociated with psittacosis*

Thirty of the 45 persons with atypical pneumonia presented no evidence of recent infection with a virus of the psittacosis group (Table I). Several samples of serum, taken at intervals after onset of pneumonia, were available from 23 of the 30 patients; none of these sera contained appreciable amounts of antibodies which reacted with psittacosis antigen. Lung tissue obtained at post-mortem examination from 2 of the 30 persons, and throat washings and sputum from another patient who recovered, all failed to yield a virus of the psittacosis group despite persistent efforts of the type which had proved successful with material

from other patients. Single specimens of serum, drawn during the second month of convalescence, were available from 5 patients; these also gave negative results in our test for antibodies of psittacosis.

It is axiomatic in virus research that a negative result, *i.e.*, the failure to isolate a viral agent or to demonstrate an immune response in a host, is less significant than a positive result. Therefore, one can only say that it is highly unlikely that 25 patients (23 from whom several samples of serum were obtained and 2 who died) of the group had an infection with the virus in question. Discussion might arise concerning the validity of the assumption that psittacosis was unrelated to the illness of those 5 individuals whose serum was examined only between the 4th and 8th weeks. It is our experience, as well as that of others (35), that complement fixing antibodies generally persist in high titer for many weeks after infection with the virus of psittacosis. Therefore, we feel justified in our opinion that a negative result in the psittacosis test on serum taken rather late in convalescence is of considerable value in ruling out the diagnosis of psittacosis. This is in contrast to the diagnostic value that may be assigned to the demonstration of psittacosis antibodies in a single sample of blood, drawn during this same period of recovery.

Serological investigations, undertaken to determine the incidence of influenza A and B and of lymphocytic choriomeningitis in the entire group of patients with atypical pneumonia, gave essentially negative results in all except 2 instances. None of the 37 patients on whom adequate material was available possessed demonstrable antibodies against antigen of choriomeningitis. Both of the persons who showed deviations from the normal levels of influenza antibodies were encountered in the group of patients in whose disease psittacosis was not a factor. Serum of one of these, M. B., had a high titer of antibodies for influenza B virus (1:768) as early as the 9th day, and this level did not change materially during the 10 weeks of observation. Serum from the other patient, T. L., showed an influenza A antibody level of 1:364 on the 6th day, and 1:64, four weeks later. It is possible that these 2 individuals had recent infections with the viruses of





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influenza B and A respectively, but the data are insufficient to prove that such infections were responsible for the pulmonary disease.

### *Epidemiological observations*

The source of infection was clearly evident in 2 of the patients with psittacosis. Both A. G. and E. G. handled acutely ill pigeons during the course of an epizootic disease in their flock, which was caused by a virus of the psittacosis group. The incubation period of the disease in both patients was approximately 3 weeks; a more detailed description of the human and avian infection will be reported (36). Similarly, patient J. M. in all probability derived his psittacosis from pigeons. Two of 6 birds in J. M.'s flock had complement fixing antibodies that reacted with psittacosis antigen. Therefore, a latent infection was undoubtedly present among the pigeons even though none of the birds was obviously sick. The occasional exposure of patient R. L. to pigeons occurred when he visited a friend while the latter "flew" his birds; this may have been the source of his infection but none of the pigeons was available for examination.

It seems worthwhile to record certain observations on the direct and indirect exposure of patients with atypical pneumonia, to birds, some of which had latent psittacosis. These data are presented only to emphasize the need for further study, since no conclusions may be drawn at this time. Three additional patients among the 10 who had psittacosis, and one patient in the group of 5 who may have had this disease, were either in direct contact with birds or had a remote connection with them, preceding the onset of atypical pneumonia. Mention need hardly be made of patients B. T. and C. M., see Table I, who were exposed to apparently healthy canaries. This avian species has been known to transmit infection to man; moreover, B. T. visited bird stores to purchase food for her canaries and may have been exposed to classical psittacine strains of the virus. The epidemiological importance of indirect exposure over long periods of time to pigeons with latent psittacosis must be considered. For example, E. Gu. and J. W. lived nearby flocks of pigeons but neither person handled the birds or even visited the lofts. It must be borne in mind

that not all patients who are associated with birds have atypical pneumonia and psittacosis, *viz.*, G. E. and M. M. in Table I.

Finally, the question arises concerning the source of infection of 3 proved cases of psittacosis and of 4 cases of atypical pneumonia who may have had psittacosis, since none of these individuals had any known contact with birds. Of course, numerous flocks of pigeons are present in all metropolitan areas and previous observations (21, 24), as well as present ones, indicate a high degree of parasitism of such birds with strains of the virus of psittacosis. Therefore, it is possible, as Meyer, Eddie, and Yanamura suggest (21), that these individuals were infected by inhaling contaminated material during transient indirect exposure to acutely ill or latently infected pigeons. On the other hand, it appears desirable to search for a humanized strain of virus of this general group which is moderately contagious for man and which is transmitted from man to man by way of the upper respiratory route. A family of viruses, as widely disseminated throughout the avian, mammalian, and human species as is the psittacosis-lymphogranuloma venereum group of agents, might be expected to possess a member with potentialities of this type.

Differences were noted in the age and sex distribution of our cases with psittacosis and with atypical pneumonia of unknown etiology. The average age of the patients with proved or suspected psittacosis was 49 years, and 9 of the 15 individuals were females. In contrast, the average for the patients with atypical pneumonia unassociated with psittacosis was 31 years, and only 11 of the 30 persons were females. In this small series of cases of atypical pneumonia, the incidence of psittacosis among pigeon fanciers, most of whom are men, and among middle aged women, many of whom are fond of pets, is impressively high.

### DISCUSSION

The present work indicates that an appreciable number, approximately one-fourth, of the sporadic cases of atypical pneumonia are caused by infection with strains of the virus of psittacosis. It also indicates that about two-thirds of the patients present no evidence of infection with members of

the psittacosis group of viruses. Diagnostic difficulties are encountered among the patients who early in the course of their disease possess antibodies that fix complement in the presence of psittacosis antigen, but who develop no notable increase in the titer of these antibodies during convalescence. At the present time, one is not justified in assuming that infection with strains of the virus of psittacosis is responsible for the pneumonic disease in this group of patients. Our observations on sera of patients with lymphogranuloma venereum are in agreement with those of Rake, Eaton and Shaffer (16), *viz.*, antibody in the sera of such individuals fixes complement with psittacosis antigen. Accordingly, rigid criteria have been established which must be fulfilled for each patient before we are willing to say that psittacosis virus is of etiological importance in that person's illness.

In this paper, the term "psittacosis" has been applied to the disease of the patients with atypical pneumonia caused by a psittacosis-like virus. Furthermore, reference has been made repeatedly to infection with strains of the virus of psittacosis. These expressions have been used because we think that the viruses isolated in our laboratory from human beings and from pigeons have so much in common with classical psittacosis virus that they are merely strains of this agent. Similarly, we would designate the viruses described by others as meningopneumonitis (19), pneumonitis (13, 16), and ornithosis (21) as strains of the virus of psittacosis. Certainly, the differences between the agents just mentioned are no greater than those found among the strains of a number of viruses. As an example, one need only mention the virus of vaccinia with its numerous strains, some of which differ strikingly from the classical agent isolated from lesions of cowpox. If only the strains of virus discussed above constituted a specific group, then there might be justification for dividing the human disease which they cause into psittacosis and ornithosis, as Meyer has done (21, 22). However, we must reckon with the viruses of lymphogranuloma venereum and of mouse pneumonitis (37) (and undoubtedly others yet to be discovered), for they too belong in the family. Therefore, until sufficient data have accumulated to warrant a reason-

able taxonomic approach to this confusing problem, it seems preferable to employ the term psittacosis-lymphogranuloma virus for this entire group, just as the expression vaccinia-variola virus is applied to that group of agents. Furthermore, until the situation is clarified, it appears reasonable to speak of the members of the family as strains of the viruses of psittacosis and of lymphogranuloma venereum, depending upon which one of these the agent in question resembles more closely.

#### SUMMARY

Ten of the 45 sporadic cases of atypical pneumonia encountered among large eastern urban populations were caused by infection with strains of the virus of psittacosis. Five additional cases in the series may have had psittacosis but the diagnosis was indefinite because of the failure to demonstrate a significant rise in antibody titer in the convalescent sera of these patients. In no instance was the virus of lymphocytic choriomeningitis associated with the illness of individuals in the group, and in only two instances was influenza virus even suspected as a possible etiological agent.

The importance of establishing an etiological diagnosis in cases of atypical pneumonia is emphasized and the essential laboratory data necessary for such a diagnosis are discussed.

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# BENCE-JONES PROTEINEMIA IN MULTIPLE MYELOMA<sup>1</sup>

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The term "Bence-Jones protein" refers to an ill-defined group of proteins having a molecular weight of approximately 37,000 (1), and the characteristic properties of precipitating out of solution when warmed to 45 to 58° C. and of redissolving completely or partially on boiling. This peculiar behavior on heating has long served as a useful test for Bence-Jones proteins in the urine of patients with multiple myeloma. When applied to serum, however, particularly to those sera where hyperproteinemia is associated with myeloma, the heat test has been found generally inadequate because it does not sufficiently differentiate Bence-Jones proteins from the serum euglobulins (2, 3). More convincing evidence of Bence-Jones proteinemia can be obtained by extraction (4, and others), protein solubility curves (5), and ultracentrifugation (6, 7, and others). However, to establish satisfactorily the frequency and degree of Bence-Jones proteinemia in multiple myeloma, and its relation to hyperproteinemia in that disease, more adequate methods recently devised for characterizing proteins would appear to be necessary. Any generalizations so derived should be based upon a series of cases large enough to be representative.

In a recent study combining electrophoretic with salting-out techniques (8), it was found that 38 cases of multiple myeloma, presenting the most varied quantitative and qualitative differences in serum proteins, could be classified into 3 major groups: 1. Those with hyperglobulinemia due to  $\gamma$  components which precipitated out chiefly with the Howe "euglobulin," and partly with the Howe "pseudoglobulin I" fraction. 2. Those with a variety of unusual serum protein patterns, not encountered in any other disease. 3. Those with apparently normal serum proteins. It was suggested that the protein increment in the first group

contained little or no Bence-Jones protein, whereas the anomalous distributions in serum protein fractions in the second group appeared to be due, for the most part, to significant Bence-Jones proteinemia. This view was based largely upon comparisons of the solubility characteristics and electrophoretic mobilities of urinary Bence-Jones proteins with those of serum proteins in multiple myeloma, and upon a study of the properties of proteins in normal serum to which different urinary Bence-Jones proteins had been added. Further and more direct evidence on this point has since been obtained by correlating the results of salt precipitation, electrophoretic and ultracentrifugal analysis, and immunological methods in myeloma serum and urine.

## METHODS

*Fractional precipitation with neutral salts.* Howe's method (9) was used for serum. Our results in normal and myeloma sera, and the uniform pattern in the distribution of protein fractions found in hyperproteinemia due to chronic infections and cirrhosis, have already been described (8).

*Electrophoresis.* The electrophoretic analyses and separations were made by means of the Tiselius apparatus (10) using a cell of 10 ml. capacity, and the Toepler Schlieren optical arrangement as modified by Longsworth (11). The samples of sera were diluted 1:4, 1:6, or 1:8 (depending upon the protein concentration) with 0.02 M phosphate buffer containing physiological saline solution at pH 7.4, the ionic strength of the buffer being  $\mu = 0.2$ . They were then dialysed against 2 or more changes of buffer for a period of 2 or 3 days, the last change of buffer being used to fill the electrode vessels. Undiluted urine samples were dialysed in the phosphate buffer ( $\mu = 0.2$ , not 0.1 as previously (8) stated) in the same manner and studied in the Tiselius apparatus.

Our experience with electrophoretic analyses of normal sera has been summarized elsewhere (12). Results with blood plasma or serum in multiple myeloma have been recorded by Longsworth, Shedlovsky and MacInnes (13), by Kekwick (14) and, in both serum and urine, by ourselves (8).

*Ultracentrifugation.* The analyses of serum and urine were made in an air-driven ultracentrifuge kindly placed

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at our disposal by Dr. A. E. Severinghaus. An analytical cell holding 0.4 ml. of the type described by Bauer and Pickels (15), was used, the optical analysis being made by means of the Toepler Schlieren method (16). The sedimentation rates were calculated from photographs taken at 5 minute intervals while the rotor was held at a constant speed of 46,400 R.P.M., giving a centrifugal field at the center of the cell of 159,000 times gravity. The experiments were done at room temperature and the sedimentation rates corrected to pure water at 20° C.

*Immunological.* The general plan followed was that of Hektoen (17), with modifications. Rabbits were immunized with Bence-Jones proteins prepared from urine, the antisera were absorbed with normal human serum to remove nonspecific antibodies, and the specific antisera so obtained were used for qualitative and quantitative precipitin tests for Bence-Jones proteins in myeloma serum. To obviate difficulties arising from serological differences in the Bence-Jones proteins of different patients (18, 19), tests for Bence-Jones protein in serum were made with antiserum prepared only from the urinary Bence-Jones protein of that same patient. Immunological studies were confined therefore to cases of multiple myeloma with Bence-Jones proteinuria.

Our detailed procedure was as follows: Unacidified urine of the patient to be tested was treated with ammonium sulfate to 40 per cent saturation, the slight precipitate which formed was rejected, and the concentration of ammonium sulfate brought to 60 per cent to precipitate the Bence-Jones proteins. The precipitate was centrifuged off, dissolved and reprecipitated once, redissolved in water and dialysed in the cold until the dialysate no longer reacted with Nessler's solution.

Rabbits were injected 4 times weekly with 1 to 5 mgm. of this Bence-Jones protein in the form of an alum precipitate (20), several courses of 16 injections each being necessary to produce antisera of sufficient titer (even then, the best of these were much weaker than those obtainable with serum proteins or with egg albumin). The rabbits were bled 5 days after the last of a course of injections.

Antibodies to the serum proteins were removed by addition of small amounts of undiluted normal human serum until no further precipitation with normal serum occurred. The precipitin tests were set up by adding 0.15 ml. of the absorbed rabbit antiserum to 0.15 ml. of appropriate dilutions of antigen (the patient's serum or urine, control serum or urine, etc.). The tubes were shaken, incubated

TABLE I

*Serum and urinary proteins in 7 cases of multiple myeloma; data obtained by the Howe method, electrophoresis, and ultracentrifugation*

	Case	Sex, age	Ma- terial	Howe fractionation							Electrophoretic fractionation										Sedimentation rate of main component
				Total protein	Albu- min	Glob- ulin	Euglob- ulin	Pseudoglobulin			Mobilities ( $U \times 10^5$ )					Concentrations					
								Total	I	II	A	$\alpha$	$\beta$	M	$\gamma$	A	$\alpha$	$\beta$	M	$\gamma$	
Group 1	1	M50	Serum Urine	13.6 0.5	3.6	10.0	7.2	2.8	2.0	0.8	5.2 5.4	3.6	2.9		0.5	26 5	5	9		60	$S_{20}$ 7.1 3.4
	39	M61	Serum Urine	13.1 1.7	3.1	10.0	5.4	4.6			4.7 5.0	3.4	2.6		0.3	20 5	4	5		71	7.1 3.4
	40	M84	Serum Urine	9.6 Trace	2.6	7.0	5.4	1.6	0.8	0.8	5.0	3.6	2.6	1.4	0.7	31	8	7	51	3	6.5
Group 2	11	M63	Serum Urine	8.1 0.9	6.0	2.1	0.1	2.0			5.4 5.4	3.5	2.8 2.8	1.6	0.5	40 5	5	13 95	36	6	4.0 2.8
	41	M73	Serum Urine	5.3 0.0	1.7	3.6	0.2	3.4	1.6	1.8	5.1	3.4	2.6		1.0	29	23	33		15	3.0
	42	F52	Serum Urine	10.5 0.0	2.9	7.6	0.3	7.3	0.8	6.5	5.4	3.3	2.5	1.7	0.1	22	10	18	46	4	7.0
Group 3	43	M51	Serum Urine	5.5 1.9	4.2	1.3	0.1	1.2	0.7	0.5	5.4 5.1	3.7	2.6 2.4		0.9	68 5	10	17 95		5	3.4

Cases 1 and 11 were reported in part elsewhere (8), the electrophoretic patterns and data in Case 11 there given having been obtained with barbiturate buffer of lesser ionic strength.

The electrophoretic component "M" refers to an extra boundary appearing between the  $\beta$  and  $\gamma$  components in the serum and in the urine of many cases of multiple myeloma.

The sedimentation constants in the last column refer to electrophoretically separated components (see text).

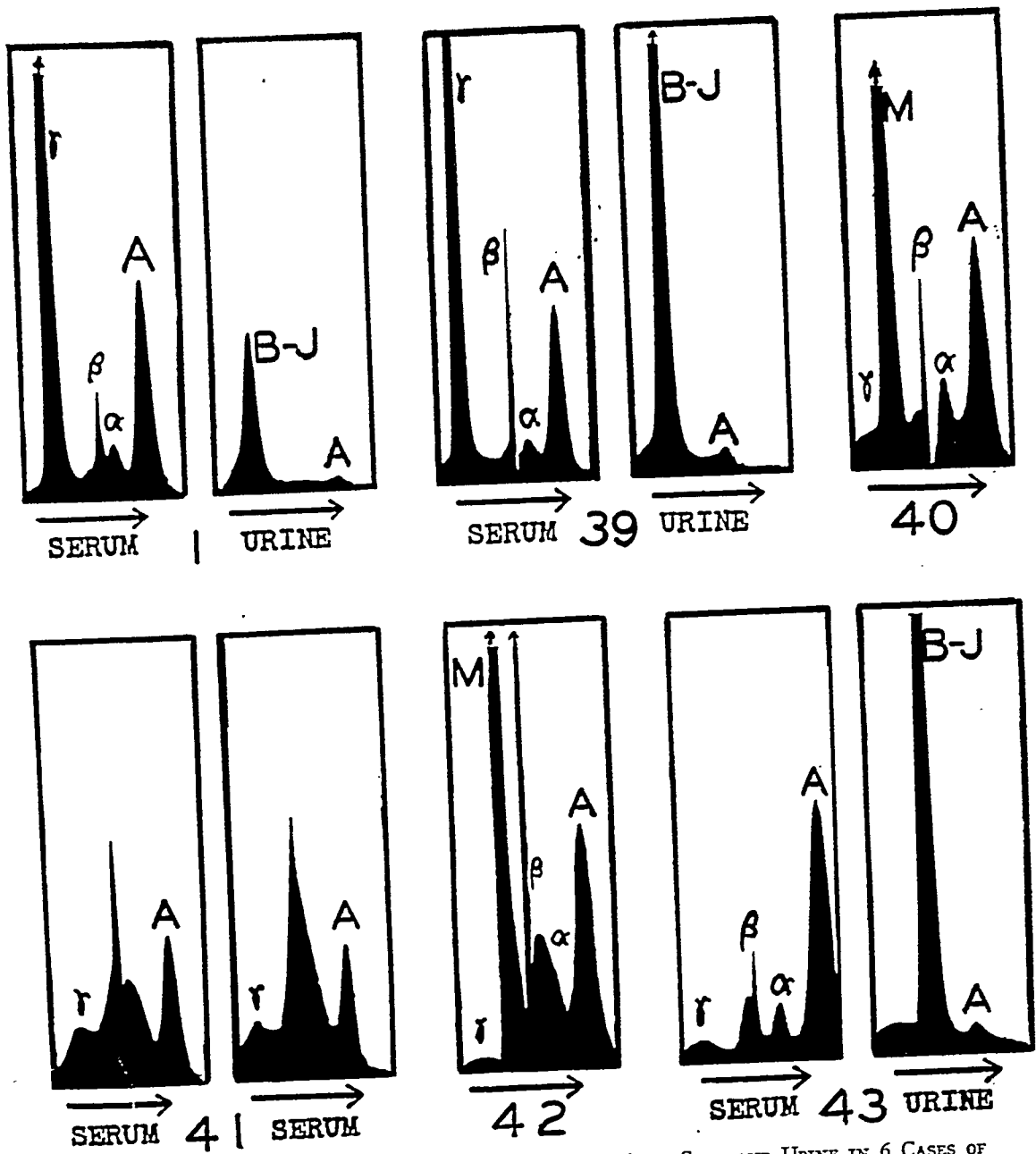


FIG. 1. ELECTROPHORETIC PATTERNS (DESCENDING LIMB) IN SERA AND URINE IN 6 CASES OF MULTIPLE MYELOMA

In Case 41, the pattern on the left was obtained before extraction of lipids, that on the right, after extraction. The pattern in Case 11 was given elsewhere (8).

at 37° C. for 2 hours, and allowed to stand overnight in the ice-box. Readings were then made in the usual manner. The amount of Bence-Jones protein present in the serum tested could be estimated roughly from the maximal dilution of antigen which still gave a precipitate. More accurate estimates were made by applying the quantitative precipitin method of Goettsch and Kendall (21). A curve was constructed representing the nitrogen content of

washed precipitates obtained by adding known amounts of purified Bence-Jones protein to measured volumes of rabbit antiserum. From this curve, by adding an appropriate amount of an unknown sample to the same volume of serum, and determining the nitrogen content of the washed precipitate, the amount of Bence-Jones protein present in unknown mixtures, such as myeloma serum, could be estimated.



## RESULTS

In Table I are summarized the results of serum protein partitions by the Howe and electrophoretic techniques in 7 cases of multiple myeloma. The diagnosis was established by autopsy or biopsy in all but Case 41. The cases are divided into the 3 major groups already described.

*Group 1.* The serum protein pattern in Cases 1 and 39 is representative of that found in a large number of patients with multiple myeloma. There is marked hyperproteinemia, the protein increment being composed of globulins, the major part of which are thrown down in 13.5 per cent sodium sulfate. The electrophoretic pattern is characterized by a large peak representing a marked increase in  $\gamma$  globulins (Figure 1), the mobilities of the main component in these 2 cases being 0.5 and 0.3 respectively.

The following evidence that the protein increment in this group of cases includes little or no Bence-Jones proteins has already been offered: 1. The distribution of serum protein fractions corresponds, in general, to the pattern uniformly found in hyperglobulinemia due to chronic infections or

cirrhosis, in which it may be presumed that Bence-Jones proteinemia does not occur. 2. Bence-Jones proteins in native urine rarely if ever have the solubility characteristics of serum euglobulins in either ammonium or sodium sulfate solution. 3. We have been unable to reproduce a large increase wholly or very largely in the Howe "euglobulin" fraction by adding various urinary Bence-Jones protein preparations to normal serum. 4. The electrophoretic pattern of normal serum, to which has been added urinary Bence-Jones protein from patients in this group, shows a new peak corresponding to the mobility of the urinary Bence-Jones protein added, but not in the  $\gamma$  range (except in the few instances in which the mobility of the urinary Bence-Jones protein is extremely low).

When the  $\gamma$  components of the sera in Cases 1 and 39 were separated electrophoretically and their sedimentation constants determined, the value obtained for  $S_{20}$  was 7.1 in both instances (Table I). The sedimentation constant of the urinary Bence-Jones proteins in both patients was 3.4  $S$  (Svedberg (22) reports values of 2.8  $S$  and 3.7  $S$  in different Bence-Jones protein preparations). It is evident that the main component in the sera was

TABLE II

*Precipitin reactions of absorbed rabbit antisera to Bence-Jones proteins with homologous urines and sera, and with normal sera*

Case I				Case II				
Antigen	Dilution	$R_{12}$ absorbed with normal human serum	Saline	Antigen	Dilution	$R_{27}$ absorbed with normal human serum	$R_{27}$ absorbed with Case 11 serum	Saline
Bence-Jones protein	1 : 10,000	+++	—	Bence-Jones protein	1 : 10,000	+++	—	—
Case 1, urine	1 : 10	++++	—	Case 11, urine	1 : 10	—	—	—
	1 : 100	+	—		1 : 25	++		—
	1 : 1000	±	—		1 : 100	+++		—
					1 : 125	+++		—
Case 1, serum	Undil.	—	—		1 : 1000	±		—
	1 : 5	+	—	Case 11, serum			—*	—
	1 : 25	++±	—		1 : 25	±		—
	1 : 125	+	—		1 : 125	++		—
Normal human serum	Undil.	—	—		1 : 625	+++		—
	1 : 5	—	—	Normal human serum				—
	1 : 25	—	—		1 : 25	—		—
	1 : 125	—	—		1 : 125	—		—
Saline		—		Saline		—		

\* 1 : 500 dilution.

of far greater molecular size than Bence-Jones protein, of the order of magnitude of normal  $\gamma$  globulins, which have been found to have a sedimentation constant of 7.1  $S$  (23). Similar results were obtained in 4 of 5 myeloma sera subjected to ultracentrifugal analysis by Kekwick (14), and in isolated cases by others. The available data suggest that Bence-Jones protein does not constitute the main protein increment in the majority of cases of multiple myeloma with marked hyperproteinemia.

While it was thus shown that the chief protein constituent of these sera was not Bence-Jones protein, it was possible by serological methods to demonstrate that Bence-Jones protein was present in small amount in the serum of Case 1. As indicated in Table II, rabbit antiserum to this patient's urinary Bence-Jones protein gave a strong precipitin reaction with a 1:25 dilution of the patient's serum, and a definite reaction was obtainable with a dilution of 1:125. These reactions cannot be attributed to normal serum protein components because normal serum failed to give any test with the (absorbed) antiserum.

It was further possible, by serological methods, to estimate the amount of Bence-Jones protein present in the serum of Case 1. Figure 2 shows the calibration curve obtained by the addition of increasing amounts of Bence-Jones protein N to 1.0 ml. of rabbit antiserum. When 1.0 ml. and 2.0 ml. of a 1:25 dilution of the serum of Case 1 were added, the N contents of the precipitates (points A and B, respectively, on the curve) corresponded to 0.013 and 0.019 mgm. of Bence-Jones protein N. Assuming a conversion factor of 6.25, this is the equivalent of 0.2 and 0.15 gram Bence-Jones protein per 100 ml. undiluted serum. It is obvious that this concentration of Bence-Jones protein, some 1.5 per cent of the total protein content of the serum, could not be detected by ultracentrifugal or electrophoretic methods.

Case 40 differs from the other members of Group 1. While the Howe partition showed marked hyperglobulinemia due to a large increase in the "euglobulin" fraction, electrophoretic analysis revealed the mobility of this component to be 1.4, considerably above the upper limits of variation in  $\gamma$  globulins (12). The sedimentation constant of the electrophoretically separated M and  $\gamma$  com-

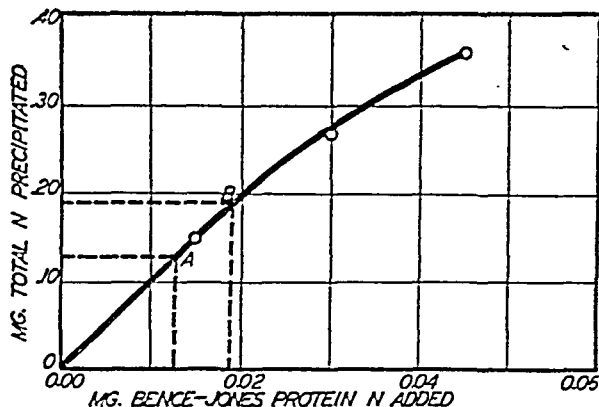


FIG. 2. CALIBRATION CURVE FOR THE DETERMINATION OF BENCE-JONES PROTEIN IN SERUM BY THE QUANTITATIVE PRECIPITIN METHOD

A blank value of 0.02 mgm. N per ml. serum without added antigen was obtained and subtracted.

All supernatants tested show a zone in which addition of either antigen or antibody gave some additional precipitation. This suggests the presence of several immunologically different components and may be related to the observation of Hektoen and Welker (19) that 2 serologically distinct Bence-Jones proteins may occur in the urine of a single patient.

ponents was found to be 6.5  $S$  (Table I). This value would indicate a molecular size far greater than that of Bence-Jones protein, at least if uncombined with other constituents. The main protein component of the serum in Case 40 was not further identified, and the classification of the case in Group 1 is tentative.

**Group 2.** The serum protein patterns in this group are varied, but fall into certain distinct categories (8): 1. Due to the presence of abnormal protein constituents which (like serum albumin) are soluble in 21.5 per cent sodium sulfate, the values for albumin, as determined by the usual Howe technique, may be falsely high; if the concentration of these constituents is sufficiently great, the results of salt precipitation may seem to indicate distinct hyperalbuminemia. 2. The Howe globulin fraction may be markedly increased, due solely to a rise in the fraction precipitable in 17.4 per cent sodium sulfate (the Howe "pseudoglobulin I" fraction) with no accompanying rise in the Howe "euglobulin" fraction. 3. The fraction precipitable in 21.5 per cent sodium sulfate may greatly exceed the normal maximum, with hyperglobulinemia due partly or solely to protein constituents having solubility characteristics corre-

sponding with those of the Howe "pseudoglobulin II" fraction.

The electrophoretic patterns in this group, likewise varied, indicate the presence of abnormal components with the mobility of  $\beta$  globulins, of  $\gamma$  globulins, or with intermediate mobilities forming a distinct intermediate boundary which we have designated as M.

The following considerations led us to suggest that these different serum protein patterns may be due, for the most part, to the presence of significant concentrations of Bence-Jones proteins, characterized by different solubilities in sodium sulfate and by different electrophoretic mobilities (8): 1. The several patterns are peculiar to multiple myeloma, the only disease in which significant Bence-Jones proteinemia may be presumed to occur. 2. The solubilities of urinary Bence-Jones proteins in sodium sulfate solution vary widely in different cases of multiple myeloma, but generally fall within the precipitation limits prescribed by Howe for serum albumins and pseudoglobulins I and II. 3. Addition of urine containing Bence-Jones protein to normal serum results in large increases chiefly in the Howe albumin, "pseudoglobulin II" or "pseudoglobulin I" fraction, depending upon the particular urine added. The inference seems justified that if such Bence-Jones proteins were present in the serum of myeloma patients and the same solubilities with respect to sodium sulfate were shown, the various Howe serum protein partitions included in Group II could result. 4. The electrophoretic mobilities of urinary Bence-Jones proteins from different patients differ widely, from 3.1 to 1.8 in the cases we have examined, *i.e.*, over the range observed in the mobilities of the extra components encountered in myeloma sera. 5. The electrophoretic patterns of normal serum to which urinary Bence-Jones proteins have been added show new  $\beta$ ,  $\gamma$ , or M peaks, according to the mobility of the particular Bence-Jones protein added. It was possible in this way to reproduce the several electrophoretic patterns characteristic of Group 2; by adding the urinary Bence-Jones protein of a given patient to normal serum, the electrophoretic pattern of that patient's serum could be duplicated (8).

Case 11 is an example of apparent hyperalbuminemia in multiple myeloma (Table I). Corre-

lation of the results of analyses by salting-out, electrophoretic, and precipitin techniques indicated that the Howe values for albumin in this case were much too high, owing to the presence of about 2.0 grams per cent of a protein with the solubility characteristics and electrophoretic mobility of the Bence-Jones protein in this patient's urine (8).

The  $\beta$  and  $\gamma$  components of the serum in Case 11 were separated electrophoretically from the  $\alpha$  globulins and albumin, and the sedimentation constant determined.<sup>2</sup> The value obtained for the main component in the ultracentrifuge, 4.0 S, cannot refer to serum albumins, which had been removed electrophoretically, and indicates a molecular size definitely smaller than that of any serum globulin. The sedimentation constant of the Bence-Jones protein in the urine of Case 11 was 2.8 S.

That the main abnormal component of the serum in Case 11 was, in fact, Bence-Jones protein in some form, could be demonstrated by serological methods (Table II). Rabbit antiserum to this patient's urinary Bence-Jones protein gave a strong precipitin reaction with a 1:625 dilution of the patient's serum. This reaction appeared to be due entirely to Bence-Jones protein since, after absorption of the rabbit antiserum with Case 11 myeloma serum, no additional precipitin reaction could be obtained with the patient's urine or purified Bence-Jones protein (Table II); nor did the (absorbed) antiserum react with normal serum protein components. Estimation of the concentration of Bence-Jones protein in the serum, by means of qualitative dilution tests, gave results of the same order of magnitude as were obtained for the main

<sup>2</sup> The separated fraction of Case 11, which included a small amount of normal  $\gamma$  globulin and an unknown amount of normal  $\beta$  globulin, showed a small, very heavy component ( $S=20$ ) but only one boundary in the range where globulins and Bence-Jones protein would be expected. The precision of the sedimentation constant determination for the lighter fractions would thus be reduced, the resulting value tending to be high for whatever Bence-Jones protein was present. Electrophoretic separation of the  $\gamma$  globulin in this fraction (only about 6 per cent, of which at least half was due to the salt boundary) was not possible because of the limited quantity of material available. Separation of Bence-Jones protein from  $\beta$  globulin in this serum would not be possible electrophoretically.

abnormal component by other methods. Quantitative precipitin determinations could not be made in this case because of the weak rabbit antisera produced and the limited amounts of urinary Bence-Jones protein available.

Case 41 showed moderate hyperglobulinemia, due to protein constituents which had the solubility characteristics in sodium sulfate of Howe's "pseudoglobulin II" fraction (Table I). The electrophoretic pattern revealed a large component moving with the mobility of  $\beta$  globulin (Figure 1). To eliminate the possibility that this might be a lipoidal substance, the serum was extracted with alcohol-ether mixture at  $-12^{\circ}$  (24). The resulting electrophoretic pattern showed no significant change (Figure 1). The cholesterol content of the serum did not exceed 118 mgm. per cent.

The  $\beta$  and  $\gamma$  components of the serum in case 41 were separated out electrophoretically and the sedimentation constant determined to be 3.0 S, a figure subject to the limits in precision already indicated. This value is consistent with a protein of the molecular size of Bence-Jones protein.

No Bence-Jones protein was found in the urine of Case 41 on repeated examination. The absence of Bence-Jones proteinuria in this patient with Bence-Jones proteinemia suggests that the serum Bence-Jones protein might have been present in the circulating fluids in the form of some complex. Bott and Richards (25) have shown that the "intact" amphibian glomerular membrane is partially permeable to proteins of Svedberg's 35,200 molecular weight group; in the case of several Bence-Jones preparations tested, the glomerular filtration values were 20 to 48 per cent. It is likely that the maximal pore diameter of the "intact" human glomerular membrane is similarly sufficient for partial passage of uncombined Bence-Jones protein in serum, since Bence-Jones proteinuria without significant albuminuria is commonly observed. If so, the absence of Bence-Jones protein in the urine of patients who give evidence of appreciable concentrations in the serum, and who have normally permeable glomerular membranes (no marked nitrogen retention), would imply that the Bence-Jones protein might occur in the serum in complexes of larger molecular size.

In the course of certain routine determinations

in the serum of Case 41, it was observed that although the serum itself was clear, the trichloroacetic acid filtrates obtained were cloudy, due to the presence of a substance which could not be removed by multiple filtration. (This phenomenon indeed gave the first intimation of anything unusual about the then undiagnosed case.) It developed that if undiluted serum were used, perfectly clear filtrates could be obtained with either tungstic or trichloroacetic acids; whereas, if the serum were first brought to the usual dilutions with water, none of the common protein precipitation gave clear filtrates. This unusual behavior would not be inconsistent with the presence of a complex dissociable on dilution. The extreme lability of this complex precluded its further study in an undissociated state.

The serum in Case 42 is unique in our experience. It contained very large amounts of a globulin which precipitated out with the Howe "pseudoglobulin II" fraction and which, migrating with the mobility of 1.7, produced an M peak intermediate between the  $\beta$  and  $\gamma$  components. The sedimentation constant of the electrophoretically separated M and  $\gamma$  components however was 7.0 S, indicating a molecular size of normal  $\gamma$  globulins. Further studies of this constituent of the serum in Case 42 have been carried out by Shapiro, Ross, and Moore (26). The main component of the serum in this instance was clearly not Bence-Jones protein and the classification of Case 42 in Group 2 is tentative.

*Group 3.* Case 43 presented normal Howe and electrophoretic serum protein patterns (Table I) and is representative of the large number of cases of multiple myeloma that show no abnormalities when studied by these methods. The urine in Case 43 contained large amounts of a Bence-Jones protein which was found to have a sedimentation constant of 3.4 S.

It was pointed out elsewhere (8) that some myeloma sera with normal Howe partitions may show abnormal electrophoretic patterns, characterized by a small M peak, presumably due to Bence-Jones proteinemia. Immunological studies, which might disclose further evidences of Bence-Jones proteinemia in apparently normal myeloma sera, have not yet been made.

## DISCUSSION

Bence-Jones proteins evidently must be present in the serum of at least those cases of multiple myeloma with Bence-Jones proteinuria, since, in the absence of any indication of a renal origin, the Bence-Jones protein must be transported by the blood from the source of origin to the kidneys. Even in cases with no excretion in the urine, the possibility of significant Bence-Jones proteinemia is not excluded, because interaction in the serum may result in the formation of non-filterable complexes. The problem therefore is not so much to determine whether Bence-Jones proteinemia ever occurs in myeloma, as to devise methods adequate for its detection and measurement.

A major difficulty in the way of this objective lies in the multiplicity of Bence-Jones proteins (18, 19, 22, 27, 28) and in their correspondingly varied properties, as illustrated by the differences in solubility, electrophoretic mobility, sedimentation rate, and serological properties observed in the urinary Bence-Jones proteins in our cases of myeloma (8, and the present study). These differences, reflected in the varied serum protein patterns of patients with Bence-Jones proteinemia, necessitate a broad and flexible analytical approach. This is particularly true in view of added difficulties due to the presence in myeloma serum of normal and abnormal proteins, usually in great excess, to the probable formation of various more or less labile complexes or combinations in some cases, and to a variety of other causes.

It would appear, nevertheless, that the problem is not beyond the reach of integrated studies by salting-out, electrophoretic, and ultracentrifugal methods, supplemented by the quantitative precipitin technique. Such, at least, is the indication of the preliminary results recorded here.

With regard to the proposed classification of serum protein patterns in multiple myeloma (8), the present data support its general validity and usefulness. It need hardly be pointed out that the classification falls far short of the ideal analysis into homogenous components, and that it is incomplete. Too little is known about the occurrence in myeloma serum of abnormal proteins other than Bence-Jones protein, however, to warrant further subdivision at this time.

## SUMMARY

The serum and urinary proteins in 7 cases of multiple myeloma were investigated by correlated salting-out, electrophoretic, and ultracentrifugal techniques, supplemented by immunological methods in 2 cases.

In 2 cases with marked hyperglobulinemia due to  $\gamma$  components which precipitated out chiefly with the Howe "euglobulin" fraction, sedimentation constants of the main component of the serum indicated a molecular size of the order of magnitude of normal  $\gamma$  globulins. Application of the quantitative precipitin technique to the serum of one of these cases further revealed the presence of approximately 0.2 gram per cent Bence-Jones protein, a concentration too low for detection by other methods. A review of the available data indicates that only a very small proportion of the protein increment is Bence-Jones protein in many (probably the majority of) cases of multiple myeloma with marked hyperproteinemia.

Two cases with sera showing abnormal  $\beta$  or M components, and unusual Howe partitions not encountered in diseases other than multiple myeloma, were found by ultracentrifugal and serological analysis to have Bence-Jones protein as the chief abnormal protein component of the serum. The available data suggest that marked Bence-Jones proteinemia does occur in multiple myeloma, probably in a lesser but clinically significant proportion of cases.

Two cases of multiple myeloma with marked hyperproteinemia were found to have large concentrations of abnormal, not readily classifiable proteins with sedimentation constants indicating an approximate molecular size of normal globulins. The serum in one case of multiple myeloma was found to have apparently normal Howe and electrophoretic serum protein patterns.

In spite of the difficulties involved in the detection and measurement of Bence-Jones proteins in serum, integrated studies of the kind indicated appear to afford a promising approach to the problem.

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# THE PREVENTION OF STREPTOCOCCAL UPPER RESPIRATORY INFECTIONS AND RHEUMATIC RECURRENCES IN RHEUMATIC CHILDREN BY THE PROPHYLACTIC USE OF SULFANILAMIDE<sup>1</sup>

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In a previous paper (1), the effect of streptococcal upper respiratory infections on groups of rheumatic children under close observation in a sanatorium was reported. It was found that during a three-year period no rheumatic relapses were observed in children who escaped streptococcal upper respiratory infections. Since, however, the total number of rheumatic recurrences was small, it seemed possible that the relationship of the streptococcal pharyngitis to the reactivation of the rheumatic process might have been accidental. To rule out this possibility, it was essential to study the effect of preventing streptococcal upper respiratory infections in rheumatic subjects by some means which had no immediate influence on the rheumatic infection itself. For this purpose, sulfanilamide was chosen, as most observers agree that this drug not only fails to benefit patients with active rheumatic fever, but actually tends to increase the severity of the rheumatic symptoms (2 to 4).

On the other hand, the observations of Coburn and Moore and of Thomas and her coworkers indicated that prophylactic doses of sulfanilamide were effective in preventing streptococcal upper respiratory infections, and that rheumatic patients, so protected, escaped rheumatic relapses (5 to 9). These authors, however, did not have an opportunity of comparing the patients who were receiving sulfanilamide with a control group living under identical conditions, where exposure to Group A hemolytic streptococci could be determined. It seemed worthwhile, therefore, to study the value of sulfanilamide prophylaxis in an institution where the patients were under daily observation and careful bacteriological studies could be made.

The type of community, routine procedures, and

bacteriological methods used were the same as those previously described (1).

## PLAN OF STUDY

During two successive winters, 1940 to 1941 and 1941 to 1942, the 108 rheumatic children at Irvington House were divided into 2 groups, matched as closely as possible in regard to age, sex, number of previous rheumatic attacks, and cardiac findings. Beginning in October 1940 and continuing until the following June, half of the children were given small daily doses of sulfanilamide.<sup>2</sup> The other 54 children served as controls. During the second winter, 1941 to 1942, 54 children were given sulfanilamide and 50 served as controls. Only children who showed neither clinical nor laboratory signs of rheumatic activity received this drug.

During the winter of 1940 to 1941, 78 per cent of the 108 patients were cases of possible and potential heart disease and 22 per cent had definite cardiac lesions. During the second winter, 1941 to 1942, the percentage of children in the group with organic heart disease was increased to 49 per cent.

## Dosage of sulfanilamide

During the winter of 1940 to 1941, an average blood level of sulfanilamide of 2 mgm. per cent was maintained. In most instances, children weighing 75 lbs. or less were given 1 gram daily in 3 divided doses, at 8 a.m., 2.30 p.m., and 8.30 p.m. Children weighing 75 lbs. or more received 1.3 to 2 grams daily.

During the winter of 1941 to 1942, the dosage was decreased slightly so as to maintain an average blood level of 1.5 mgm. per cent. Samples of bloods for routine determinations of sulfanilamide

<sup>1</sup> This work was aided by a grant from the Commonwealth Fund.

<sup>2</sup> Sulfanilamide (Prontylin) for this study was donated through the courtesy of the Winthrop Chemical Company.



levels were taken once every 3 weeks, before the 8 a.m. dose. In a small number of cases, the sulfanilamide content obtained before the morning dose was compared with that obtained before the 2.30 p.m. or 8.30 p.m. dose, and it was found that the blood level of sulfanilamide maintained throughout the course of the day was remarkably constant.

*Streptococcal upper respiratory infections in the control group, 1940 to 1941*

In October 1940, a routine culture of one of the children in the control group showed large numbers of Group A hemolytic streptococci Type 15. This child did not complain or present symptoms or show a rise in antistreptolysin O titer. This type of streptococcus had not been present previously in the community and the source of this microorganism was not determined.

Between the end of October 1940 and the end of January 1941, 30 cases of pharyngitis associated with this type of streptococcus developed among the 54 children in the control group. As in epidemics previously reported, the spread of this upper respiratory infection was slow: 3 cases developed in October, 8 in November, 12 in December, and 7 in January. Sixteen of these 30 cases were of moderate severity, with rectal temperatures of 101° F. or more. Eight had very mild symptoms. Six children did not have complaints or symptoms; and the diagnosis of pharyngitis was based solely on laboratory data (routine throat cultures positive for streptococcus Type 15 accompanied by a rise in white blood count or followed by a rise in antistreptolysin O titer).

*Incidence of rheumatic recurrence in the control group, 1940 to 1941*

Following a latent period, varying from 3 to 21 days, 14 of these 30 children developed definite rheumatic recurrences. Three of these 14 children had originally been in the group taking sulfanilamide but were unable to tolerate the drug. They contracted the Type 15 streptococcus infection 4 days, 5 weeks, and 2 months, respectively, following the withdrawal of sulfanilamide.

Of these 14 children with definite rheumatic relapses, 4 had organic heart disease prior to these attacks and 10 did not. The rheumatic manifesta-

tions in 11 of these 14 patients (10 cases of possible and potential heart disease and 1 case of organic heart disease) were of short duration. Three children whose rheumatic symptoms persisted for several months had definite cardiac lesions.

Eight of the 14 rheumatic relapses followed pharyngitis of moderate severity. Four occurred in children who had mild upper respiratory symptoms, and 2 developed in patients in whom the diagnosis of the preceding pharyngitis was based only on laboratory data.

Four additional children of the 30 who had the Type 15 upper respiratory infection developed, following a latent period, distinct laboratory signs of rheumatic activity (leukocytosis and increased erythrocyte sedimentation rates which persisted for 1 month or more), but had no definite clinical symptoms, or changes in their X-ray or electrocardiographic findings, and therefore were classified as having questionable rheumatic recurrences. One of these children had received sulfanilamide originally but could not tolerate the drug; his routine throat culture revealed hemolytic streptococci 2 weeks after the withdrawal of sulfanilamide. He had no complaints or symptoms but his antistreptolysin O titer rose, and his erythrocyte sedimentation rate remained elevated for 6 weeks.

*Streptococcal carriers in the control group, 1940 to 1941*

Two children in the control group became carriers of streptococcus Type 15 without developing symptoms of any kind. The leukocyte count, erythrocyte sedimentation rate, and antistreptolysin O titer in these patients showed no changes.

*Streptococcal upper respiratory infections in the sulfanilamide group, 1940 to 1941*

Only one child in this group contracted pharyngitis due to streptococcus Type 15. She complained of sore throat and had a temperature ranging from 101.2 to 100° F. for 2 days; and her leukocyte count was elevated. The same dose of sulfanilamide was continued and she recovered promptly. Her erythrocyte sedimentation rate remained normal and no rheumatic sequelae developed. The sulfanilamide blood level was 2 mgm. per cent at the time of her infection.

*Streptococcal carriers in the sulfanilamide group, 1940 to 1941*

Ten children, who received the drug in whom an average blood level of 2 mgm. per cent was maintained, became carriers of Type 15 without showing either clinical or laboratory evidence of infection. It is of interest that none of these children developed pharyngitis when sulfanilamide was discontinued on June 1, 1941, although 6 of them were still carrying streptococcus Type 15 at that time.

TABLE I

*The seasonal distribution of streptococcus Type 15 upper respiratory infections and incidence of rheumatic recurrences, 1940 to 1941*

Seasonal distribution	Control group (54 children)			Sulfanilamide group (54 children)		
	Pharyngitis	Definite rheumatic recurrences	Questionable rheumatic recurrences	Pharyngitis	Definite rheumatic recurrences	Questionable rheumatic recurrences
October	3	0	0			
November	8	3	0			
December	12	5	4	1	0	0
January	7	6	0			
February	0	0	0			
Total	30	14	4	1	0	0

*Results, 1940 to 1941*

The contrast as shown in Table I between the incidence of streptococcal pharyngitis among the children in the control group and those receiving sulfanilamide was striking, 30 to 1. Not a single child who received the drug developed rheumatic manifestations, whereas 14 of the 30, or nearly half, in the control group, who had streptococcus Type 15 upper respiratory infections, showed definite rheumatic relapses, and 4 additional children had laboratory evidence suggesting a reactivation of the rheumatic process.

*Streptococcal upper respiratory infections, 1941 to 1942*

On October 21, 1941, a boy (H. B.) was admitted with mild upper respiratory symptoms. His admission throat culture was positive for Group A hemolytic streptococci of undetermined type.

Beginning October 26th and continuing until the middle of January 1942, 17 children in the control group of 50 developed pharyngitis due to a streptococcus which appeared identical with the strain isolated from H. B. Rabbits were immunized with this microorganism, designated as B35, isolated from one of these patients; and with their sera used in slide agglutination (Griffith) and anti M precipitin tests (Lancefield), the strains isolated from the 17 cases of pharyngitis, as well as the one obtained from H. B. on admission, were shown to represent a single serological type, designated as streptococcus B35 in the remainder of this paper.<sup>2</sup>

The spread of streptococcus B35 was more rapid than that observed in previous outbreaks of streptococcal pharyngitis: 6 cases occurred in October and 9 in November, followed by 1 in December and 1 in January. Thirteen of these 17 cases were of moderate severity with rectal temperatures of 101° F. or more. In 2 patients, the symptoms were mild. Two children had neither complaints nor symptoms and the diagnosis of pharyngitis was based on laboratory findings.

*Incidence of rheumatic recurrences in the control group, 1941 to 1942*

Following a latent period varying from 10 to 18 days, 9 of these 18 children developed definite rheumatic recurrences and one a questionable rheumatic recurrence. Of these 10 children with rheumatic manifestations, 6 had organic heart disease previous to these attacks and 4 were cases of possible and potential heart disease. The rheumatic recurrences in 4 of these 10 children were severe (pericardial friction rub, subcutaneous nodules, persistent high fever). Of these 4, 3 had organic heart disease prior to these attacks and one did not.

*Streptococcal carriers in the control group, 1941 to 1942*

Two children in the control group became carriers of the epidemic inducing strain, B35, without developing symptoms of any kind.

<sup>2</sup> As will be reported by E. Krumwiede, in a paper now in press, this strain has been accepted as a new type and has been given the provisional type number 36.

### *Streptococcal upper respiratory infections in the sulfanilamide group, 1941 to 1942*

Only one child in this group developed pharyngitis due to streptococcus B35. At the time of infection, the sulfanilamide level in the blood was only 0.95 mgm. per cent instead of the usual 1.5 mgm. per cent. Following a latent period, this patient developed mild rheumatic manifestations, namely, a prolongation of PR interval from 0.15 to 0.23 second, a drop in hemoglobin and red cells, and an elevated erythrocyte sedimentation rate. These symptoms lasted 10 days. There was no increase in the size of the heart nor any changes in the heart sounds.

### *Streptococcal carriers in the sulfanilamide group, 1941 to 1942*

Four children who received this drug, and in whom the sulfanilamide content of the blood was maintained at an average level of 1.5 mgm. per cent, became carriers of streptococcus B35.

TABLE II

*The seasonal distribution of streptococcus B35 upper respiratory infections and incidence of rheumatic recurrences, 1941 to 1942*

Seasonal distribution	Control group (50 children)			Sulfanilamide group (54 children)		
	Pharyngitis	Definite rheumatic recurrences	Questionable rheumatic recurrences	Pharyngitis	Definite rheumatic recurrences	Questionable rheumatic recurrences
October	7	4	0	0	0	0
November	9	4	1	1	1	0
December	1	1	0	0	0	0
January	1	0	0	0	0	0
Total	18	9	1	1	1	0

### *Results, 1941 to 1942*

As shown in Table II, the contrast between the incidence of streptococcal pharyngitis among the children in the control group and those receiving sulfanilamide was again clear cut, 18 to 1. The difference in the incidence of definite rheumatic recurrences in the two groups was 9 to 1.

### *Toxic reactions*

During the first year, 1940 to 1941, 10 of the 54 children who were started on sulfanilamide developed toxic reactions of sufficient severity to

necessitate stopping the drug, and other children were substituted for them.

During the second year, 1941 to 1942, the group receiving sulfanilamide consisted of 23 children who had taken the drug during the previous winter and 31 new patients. Among these 31 children, 5 were unable to tolerate the drug. Thus, of the total of 100 patients given prophylactic doses of sulfanilamide, toxic reactions developed in 15.

Similar toxic reactions were encountered during the two successive years: namely, fever, nausea and vomiting, skin manifestations, and leukopenia. The age, weight, dosage, and symptoms of the 15 children who developed toxic manifestations are summarized in Table III.

The most frequent toxic reaction was fever, in several instances accompanied by abdominal pain and vomiting. This reaction developed in 7 patients between the 5th and 13th day of medication after 10 to 26 grams of the drug had been given.

Five children developed skin manifestations. In 4 of them, this symptom appeared between the 11th and 15th days of medication when 11 to 14.5 grams of sulfanilamide had been taken. In 2 children, the rash was erythematous and the leukocyte count and temperature remained normal. In 2 others, the rash was urticarial and was accompanied by leukocytosis. One of these 2 children was afebrile. In the other, the urticaria was less extensive and the drug was not discontinued immediately; but 2 days later, this patient developed a temperature of 103° F. and the medication was stopped. In the 5th child, Number 9, Table III, the rash was also urticarial but did not develop until the 31st day of medication when a total of 70 grams had been taken. This patient had a temperature of 100° F. and a slightly elevated leukocyte count.

Leukopenia developed in 3 patients, after 3 to 4 weeks, when they had received 21 to 28 grams of sulfanilamide. The total number of white blood cells, as well as the percentage of polymorphonuclears, decreased gradually.

### *Results obtained by retesting patients who had developed toxic manifestations*

In order to prove that the symptoms or blood changes observed were really due to sulfanilamide, the drug was restarted in 9 of these patients after intervals of 8 days to 18 months.

TABLE III  
Toxic manifestations induced by sulfanilamide

Case number	Patient			Weight	Daily dose	Symptoms	Day of appearance	Further observations	Blood level
	Name	Sex	Age						
			years	lbs.	grams				mgm. per cent
1	S.S. 3593	M	12	77	2	Fever 102° F., WBC 10,400	5	After 8 days restarted. After 0.6 grams, fever 101° F. and vomiting.	
2	H.C. 3607	F	11	66	1	Erythema, WBC normal	11	18 months later tolerated a total of 26.7 grams given in 31 days.	1.1
3	F.S. 3529	F	8	51	1	Erythema, WBC normal	12	Patient discharged.	
4	G.R. 3463	F	11	70	1	Urticaria, WBC 20,000	15	After 18 months restarted. Urticarial rash after 8 grams had been given in 12 days, WBC 13,300.	1.05
5	R.A. 3581	F	15	104	2	Fever 100.4° F., WBC normal	13	After 12 days restarted on 1 gram daily. Rash after 7 days. WBC 11,000.	
6	P.P. 3558	F	8	55	1	Leukopenia, WBC 3,000, PMN 27 per cent	28	After 16 days restarted on same dose. Leukopenia after 23 days.	1.8
7	M.M. 3596	F	11	73	1	Leukopenia, WBC 2,700, PMN 24 per cent	22	After 6 weeks restarted on same dose. Leukopenia after 22 days.	1.4
8	D.B. 3584	M	11	89	2	Fever 101.2° F., WBC 10,300, abdominal pain	6	After 13 days restarted on 1 gram daily. Fever 101.4° F., vomiting after 16 days.	
9	J.C. 3606	F	14	110	2	Urticaria, WBC 10,500	35	Patient not retested.	
10	H.M. 3577	F	13	106	2	Fever 100.6° F., WBC 11,300	5	After 1 month restarted on same dose. Fever 105° F., WBC 12,200 on 2nd day.	2.8
11	S.C. 3669	M	13	105	1.3	Fever 102.4° F., WBC normal	9	6 months later tolerated a total of 26.7 grams given in 31 days.	1.
12	V.C. 3670	M	12	107	1.3	Fever 101.8° F., WBC normal	8	After 36 days started on sulfadiazine 1 gram daily, well tolerated.	2.6
13	S.T. 3676	M	14	111	1.3	Fever 101.2° F., abdominal pain, WBC normal	10	After 4 days started on sulfathiazole 1.3 grams daily. Fever and abdominal pain after 20 days. After 12 days started on sulfadiazine 1 gram daily. Fever and abdominal pain after 22 days.	1.25
14	A.S. 3718	F	8	54	1.	Leukopenia, WBC 2,800, PMN 33 per cent	21	After 55 days started on sulfadiazine 0.7 grams daily. Well tolerated.	1.9
15	F.A. 3722	F	13	89	1.3	Urticaria, WBC 11,900 Fever 103° F.	11 13	After 26 days started on sulfadiazine 0.7 grams daily. Well tolerated.	2.

Two patients, Numbers 1 and 10, Table III, who had developed fever, again showed febrile reactions of greater severity in a shorter period of time. A 3rd patient, Number 5, Table III, who developed fever the first time the drug was given, showed an urticarial rash without an elevation of temperature within a shorter period of time, although a smaller dose of sulfanilamide was used.

Another patient, Number 8, Table III, who had developed fever and abdominal pain, again showed similar symptoms when a smaller dose was given. This time, however, the child tolerated a somewhat larger total amount (16 grams instead of 12), and the toxic manifestations took 10 days longer to appear.

On the other hand, patient Number 11, who had shown a febrile reaction considered to be due to

the drug, was given sulfanilamide again after an interval of 6 months. Restarted on 0.3 grams a day, the dose was then gradually increased to 1 gram a day. This time the patient received a total of 26.7 grams in 31 days without developing symptoms of any kind.

Two patients, Number 2 and Number 4, who had developed rashes considered to be due to sulfanilamide, were retested after an interval of 18 months. Both children were started on 0.3 grams a day and the dose was then gradually increased to 1 gram a day. This time patient Number 2 received a total of 26.7 grams in 31 days without developing toxic manifestations; but the other child, Number 4, again developed urticaria and leukocytosis after receiving a total of 8 grams in 12 days.

Another patient, not included in Table III, is also worth mentioning. A boy developed an extensive, itching, urticarial rash after receiving sulfanilamide for  $2\frac{1}{2}$  months. He had no fever and the leukocyte count was within normal limits. This patient had no history of asthma, hay fever, or food allergy. The drug was discontinued and the urticaria disappeared within 48 hours. After 2 weeks, this boy was restarted on 0.3 gram of sulfanilamide a day. The dose was increased gradually to 1.3 grams a day and then maintained for 4 months with no untoward effect.

Two patients, Numbers 6 and 7, Table III, who had developed leukopenia, again showed similar changes in the blood picture, with the same dosage, in approximately the same length of time.

Thus of 9 children retested, 7 again developed symptoms and 2 did not. It may be that the reactions originally observed in these 2 patients were not due to sulfanilamide. On the other hand, it also seems possible that the toxic manifestations were transient and did not recur when the drug was given more slowly.

Four of the 5 children who could not tolerate the drug during the winter of 1941 to 1942 were subsequently given sulfadiazine.<sup>4</sup> One boy developed symptoms identical with those caused by sulfanilamide, namely, fever and abdominal pain. The other 3 children tolerated sulfadiazine.

#### *General condition of children receiving prophylactic doses of sulfanilamide*

The children who did not develop toxic manifestations within 5 weeks tolerated the drug well. There were no subjective complaints. In most instances, the patients continued to gain weight at the same rate as before medication was started. The weight gain in children receiving sulfanilamide was comparable to that of children in the control group. Cyanosis was noticeable only in a few light-complexioned patients.

#### *Administration of sulfanilamide for two successive winters*

Twenty-three children who had received sulfanilamide during the winter of 1940 to 1941 remained in the institution. The drug was discontinued

on June 1st, 1941 and restarted in October 1941 without inducing toxic manifestations.

#### *Minor transitory toxic reactions*

Leukocyte count and hemoglobin determinations were made once a week on every child receiving sulfanilamide. The total red cells were determined every 2 weeks, or oftener when indicated.<sup>5</sup>

It was found that the hemoglobin of most children receiving sulfanilamide tended to fall slightly and remained at a level somewhat lower than normal throughout the course of treatment. When the drug was discontinued, the hemoglobin rose to its previous level. In one instance, a boy of 12 years, weighing 79 lbs., with typical mitral stenosis, received 1 gram of sulfanilamide a day. His hemoglobin dropped from 14.5 to 10.5 grams per 100 cc. during the first week of medication. At the same time, his hematocrit reading fell from 42 to 32 per cent and his red blood count from 4,820,000 to 3,100,000 cells. He had no complaints, no jaundice, and normal urine findings. His blood sulfanilamide level was 1 mgm. per cent. Since he had no clinical symptoms and because a blood level of 1 mgm. per cent was considered insufficient to protect him from streptococcal pharyngitis, the dosage was increased to 1.3 grams a day. Within a week his hemoglobin, hematocrit reading, and red blood count began to rise. Thereafter, his hemoglobin ranged between 13 to 14 grams and his red blood count between 3,900,000 to 4,510,000 cells.

Minor fluctuations in the total leukocyte count and percentage of polymorphonuclears were also observed. In most instances, these changes were no greater than those encountered from time to time among children in the control group. An outbreak of upper respiratory infections of unknown etiology, accompanied by leukopenia, occurred in the institution during April and May 1942. With a few exceptions, sulfanilamide was not discontinued. The fall in leukocyte count in children receiving sulfanilamide and that in the control group were equally striking. However, the leukopenia tended to persist for a slightly longer period in patients receiving the drug.

<sup>4</sup> Sulfadiazine was donated through the courtesy of Lederle Laboratories, Inc.

<sup>5</sup> The expense of the technical assistance needed for the hematological studies was carried in part by a grant from The Borden Company.

## DISCUSSION

The effect on the incidence of rheumatic relapses of preventing streptococcal upper respiratory infections by the prophylactic use of sulfanilamide is reported. Small daily doses of the drug were given to half of a group of rheumatic children under close observation in a sanatorium from October until June, during 2 successive winters. The contrast between the incidence of streptococcal upper respiratory infections and rheumatic relapses in the treated and untreated groups was clear-cut. Of the 108 children receiving sulfanilamide, only 2 children contracted streptococcal upper respiratory infections, and only one of these 2 patients showed signs of rheumatic activity. Among the 104 children who served as controls, 48 contracted streptococcal pharyngitis, and 23 of these, or 48 per cent, developed definite rheumatic relapses, and 5 additional children had laboratory evidence or mild symptoms suggesting rheumatic activity. In accord with our previous experience, no rheumatic relapses were observed in children who escaped streptococcal upper respiratory infections (1).

Evidence that rheumatic relapses usually follow in the wake of upper respiratory infections associated with Group A hemolytic streptococci had been accumulating for many years. The high incidence of rheumatic fever and rheumatic recurrences following outbreaks of tonsillitis in schools and training camps, as well as in convalescent homes for rheumatic children (10 to 14), suggested that streptococci might play a role in the etiology of this disease.

It is now well known that rheumatic relapses follow mild as well as severe cases of streptococcal pharyngitis. The symptoms of the upper respiratory infection may indeed be so slight as to be completely forgotten by ambulatory patients at the time that the rheumatic manifestations appear. Even in convalescent homes, cases of streptococcal pharyngitis may be overlooked unless careful bacteriological and serological studies are made. It is not surprising, therefore, that some observers have been inclined to doubt the importance of the relationship between streptococcal pharyngitis and rheumatic fever.

With the advent of sulfanilamide, a specific means of preventing streptococcal upper respira-

tory infections in rheumatic subjects became available. The studies of Coburn and Moore, and Thomas and her coworkers, indicated that the prevention of streptococcal pharyngitis also prevented rheumatic relapses. However, nearly all the patients included in the series of Thomas, *et al.*, were more than 14 years of age, when the incidence of rheumatic recurrences tends to decline spontaneously. In Coburn and Moore's studies, most of the children were ambulatory and the degree of exposure to Group A hemolytic streptococci could not be determined so accurately as among patients living in an institution. In our series, the incidence of streptococcal upper respiratory infections was high among the children in the control group, and nearly 50 per cent of those so infected developed rheumatic sequelae. Our results are in accord with those mentioned above and suggest that in closed communities where the spread of streptococci is difficult to control, prophylactic doses of sulfanilamide are effective in preventing both streptococcal pharyngitis and rheumatic relapses. Furthermore, these studies, aside from showing the prophylactic value of sulfanilamide, indicate that the relationship between streptococcal upper respiratory infections and the reactivation of the rheumatic process is specific, and therefore establish the importance of Group A hemolytic streptococci as a factor in the etiology of rheumatic fever.

Although the course of rheumatic fever in certain individuals is insidious, severe cardiac damage is usually the result of repeated rheumatic relapses (15). The chief aim therefore of the physician who has rheumatic patients under his care, should be the prevention of rheumatic recurrences. The effectiveness of prophylactic sulfanilamide in preventing rheumatic relapses has been established by our findings as well as those of others. In considering any prophylactic measure, however, the inherent danger must be carefully weighed.

The two most serious toxic reactions which have been reported during the course of sulfanilamide therapy are: acute hemolytic anemia and acute agranulocytosis. To date, hemolytic anemia has not been described in patients receiving prophylactic doses of sulfanilamide.

One instance of a fatal acute agranulocytosis, however, has been reported by Stowell and But-

ton (16). A boy of 12 years developed this complication after receiving 0.6 gram three times a day for 29 days. Acute agranulocytosis is rare in patients receiving sulfanilamide therapy. It occurs most commonly between the 17th and 25th day of treatment but may appear as early as the 14th and as late as the 70th day, and is independent of dosage (17, 18). Acute agranulocytosis develops suddenly and probably represents a peculiar idiosyncrasy to sulfanilamide. The possibility of this complication in patients receiving prophylactic sulfanilamide must always be borne in mind and constitutes the greatest hazard of this form of treatment.

The incidence of toxic reaction in our series (15 per cent) was higher than that reported by Coburn and Moore (6). No serious reactions were encountered. Even though smaller doses were used during the second winter than during the first, the incidence of toxic reaction was not reduced. It is our impression that in most instances the development of toxic reactions is due to an idiosyncrasy of the individual, rather than to the size of the dose. Twenty-three patients who received sulfanilamide during two successive winters showed no evidence of sensitization when the drug was restarted after a lapse of nearly 5 months.

In one instance, a blood level of 2 mgm. per cent was insufficient to prevent streptococcal pharyngitis. A similar failure was encountered in a patient with a blood level of 0.95 mgm. per cent. It seems possible that the child with the level of 2 mgm. per cent who contracted the streptococcal infection, was either very susceptible to the particular streptococcus or that the infecting dose was unusually large. In most instances, a blood level of 2 mgm. per cent seems to be adequate. On the other hand, we are of the opinion that a blood level of 1 mgm. per cent is probably too low to be effective.

The ultimate value of prophylactic sulfanilamide can be determined only by protecting rheumatic individuals from streptococcal upper respiratory infections for long periods of time. The prolonged administration of any drug as toxic as sulfanilamide may eventually prove harmful even in patients who apparently tolerate the drug. Furthermore, individuals who have been protected against infection with Group A hemolytic strepto-

cocci for many years may be extremely susceptible to these microorganisms when sulfanilamide is withdrawn.

On the other hand, the prognosis in patients with severe rheumatic heart disease is so poor as to justify taking risks. In our opinion, at the present stage of our knowledge, the effect of the prolonged administration of sulfanilamide should first be tried in this type of case.

To date, no reports have appeared on the prophylactic use of sulfadiazine in preventing streptococcal upper respiratory infections; and our own experience is too limited to warrant drawing conclusions. It seems likely that this drug will prove as effective as sulfanilamide.

Although it now seems established that Group A hemolytic streptococci play a part in precipitating rheumatic recurrences, the mode of action of these microorganisms in this disease remains obscure. It is possible that as our knowledge of the immunological response of individuals to streptococcal infections increases, new methods of combatting streptococci, based on biological reactions rather than on chemotherapy, will be devised.

#### CONCLUSIONS

1. Streptococcal upper respiratory infections and rheumatic relapses in rheumatic children were prevented by the prophylactic administration of sulfanilamide.
2. Toxic manifestations of sufficient severity to necessitate the withdrawal of the drug occurred in 15 per cent of the patients.
3. Children, who did not develop toxic reactions, tolerated the drug well.
4. The effectiveness of sulfanilamide in preventing rheumatic recurrences indicates that infection with Group A hemolytic streptococci is an important factor in the etiology of rheumatic fever.

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# PHENYLPYRUVIC OLIGOPHRENIA. REPORT OF A CASE IN AN INFANT WITH QUANTITATIVE CHEMICAL STUDIES OF THE URINE

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The object of this study is to present quantitative determinations of phenylalanine, tyrosine, and some of their derivatives, excreted by a two-year-old boy with clinical and urinary findings diagnostic of the rare condition known as phenylpyruvic oligophrenia. This metabolic aberration, manifested by the excretion of phenylpyruvic acid, was first described by Fölling in 1934 (1) in 10 feeble-minded patients, and cases have subsequently been reported by Penrose (2), Jervis (3, 4), and others. The disease is characterized clinically by marked mental deficiency, frequently accompanied by athetosis and other neurological stigmata. Many of the patients are well-developed physically, and even attractive in appearance. Blond hair and complexion and a susceptibility to eczema are frequently observed. The incidence among institutionalized feeble-minded individuals of all ages is about 0.8 per cent. On the basis of extensive genetic studies, Jervis (4) confirmed Penrose's opinion (5) that the anomaly is determined by a single autosomal recessive gene.

The quantity of phenylpyruvic acid in the urine varies with the protein intake, but even on a protein-free diet some is always excreted, derived probably from body protein. The excretion of the compound is greatly increased by the administration of phenylalanine, protein (casein), or phenylpyruvic acid, and to a lesser extent by phenyllactic acid, whereas other amino acids, including tyrosine, have no effect on its output. Thus the defect is, apparently, limited to the metabolism of phenylalanine.

## REPORT OF CASE <sup>1</sup>

S. W., a boy aged 25 months, was admitted on November 25, 1941, with the chief complaints of retardation of development and eczema, after a urine specimen had given a positive qualitative test for phenylpyruvic acid.

<sup>1</sup>We are grateful to Dr. T. D. Van Orden for the privilege of observing this patient.

The family history was positive for allergic manifestations, including hives in the father, and eczema and asthma in a brother. There was no family history of nervous illness. The only sibling, a brother, had been slow in development, having walked at 2 years of age and talked at 3, but was described by his pediatrician as entirely normal at his present age of 4. Tests of the father's, mother's, and brother's urines for phenylpyruvic acid were negative. The pregnancy was complicated by severe nausea and vomiting, and by a small pelvis which necessitated delivery by Caesarian section. The patient had been subject to a severe eczematous eruption since the age of 2 months. Mental retardation had been suspected at 4 months. The infant failed to raise his head until the age of one year, and began to take notice of his surroundings and to recognize his parents at 20 months. His first tooth erupted at 6 months. He sat without support for no more than a few minutes, and did not form words. He did not reach for objects but he seemed to take pleasure in holding a toy when it was placed in his hand.

Convulsions were never observed, but the infant was said to "jump" occasionally, with momentary flexion of the head and extension of all 4 extremities.

Physical examination revealed a large, obese, flabby-muscled, blond boy of 26 months, who could sit unsupported for a few minutes and could maintain himself on his arms and knees in a crouching position. Most of the time he lay quietly on his back. He followed a light or a large object with his eyes, and responded sluggishly to sounds. He had an attractive facies with a smiling expression. He was good-natured and contented most of the time, but occasionally cried and sometimes scratched his face, arms, and legs which were the sites of a dry, scaly, patchy, eczematous eruption. The child weighed 14 kilograms, and the body measurements were as follows: head circumference 46 cm., chest 55.5 cm., abdomen 54 cm., stem length 56 cm., total length 89 cm. The head appeared somewhat small in proportion to the body, but was well-formed. The fontanelles were closed. The eyes were blue, the pupils not unusual and the fundi normal. Except for moderately hyperactive deep reflexes the neurological examination was negative, and the remainder of the physical examination revealed no abnormal findings.

Roentgenograms of the skull and wrists were within normal limits. The Kline, Schick, and Mantoux tests were negative, and routine urinalyses and blood count were

normal. The blood plasma ascorbic acid level which on admission was 0.7 mgm. per cent fell to 0.3 mgm. per cent after 9 days of low ascorbic acid intake, and rose to 0.6 mgm. per cent within 4 hours after an oral test dose of 200 mgm. of ascorbic acid.

Psychometric examination by a consultant psychologist confirmed the clinical impression of marked mental retardation.

On admission, the child was given a measured diet, low in ascorbic acid but adequate in thiamin and other vitamins, and calculated to provide 4 grams of protein per kilogram of body weight, daily. The exact amount of nitrogen consumed, including that in added amino acids, is recorded in Table I. A description of the chemical methods and a report of the urinary findings follows.

#### CHEMICAL METHODS

The method described by Hoag (6) for the quantitative collection of urine in young male infants permitted the collection of 22 12-hour urine specimens without loss. Voidings were timed to the nearest minute, the actual collection periods varying usually between 11 and 13 hours. Results were calculated in terms of 12-hour periods. The specimens were preserved with toluene.

Total urinary nitrogen was determined by the macro-Kjeldahl method.

Phenylpyruvic acid may be detected by the development of a transient but characteristic green color upon the addition of 5 per cent ferric chloride to acidified urine. This reaction forms the basis of a quantitative method, worked out by Jervis, Block, Bolling, and Kanze (7) for blood and spinal fluid filtrates, and has been adapted for the determination of the compound in urine.

Phenylalanine was determined by a further modification of the Kapeller-Adler method (8) as recently modified by Block and Bolling (9). The urine was acidified and extracted with ether for 24 hours in a continuous extractor. This procedure removed phenylpyruvic and phenyllactic acids which interfere with the determination of phenylalanine. Tyrosine was removed by treatment with potassium permanganate in the cold, as recommended by Kapeller-Adler (8).

The optical isomers of phenylalanine were determined by means of the d-amino acid oxidase. The enzyme was extracted with M/60 sodium pyrophosphate solution at pH 8.0 from acetone-dried sheep kidneys, as described by Negelein and Brömel (10). Their more elaborate procedure ("step 1") was found unnecessary; correction for the error introduced by the small amount of acetone remaining in the kidney extract was made by a suitable blank. This enzyme converts the d-portion of d,l-phenylalanine into the corresponding keto acid (phenylpyruvic). The urine to be tested was extracted with ether to remove phenylpyruvic acid and then incubated with the enzyme for 3 hours at 37° C. Proteins were precipitated with trichloroacetic acid, and phenylpyruvic acid determined in the filtrate by the dinitrophenylhydrazone method of Penrose and Quastel (11a), as modified by Sealock

(11b). From the amount of phenylpyruvic acid produced, could be calculated the d-phenylalanine in the urine, and l-phenylalanine could be calculated by difference.

Jervis and his co-workers (7) have reported that phenyllactic acid gives almost as much color in the nitration process of the Kapeller-Adler method as does phenylalanine. Consequently, the method was used for the determination of phenyllactic acid as follows: To the urine was added an equal volume of saturated sodium bisulfite solution. This formed an addition compound with phenylpyruvic acid which was insoluble in ether. The urine-bisulfite mixture was then made just acid to Congo red with sulfuric acid and extracted with ether for 24 hours in a continuous extractor. The ether extract, containing phenyllactic acid, but no phenylalanine or phenylpyruvic acid, was then used for the determination of the former acid by the Kapeller-Adler method. For the purposes of this study, it appears reasonable to assume that the compound determined was actually phenyllactic acid, but complete verification must await isolation of the pure compound from the urine. If error exists, it is presumably in the direction of making the reported amounts of phenyllactic acid too high.

The Millon method as modified by Folin and Ciocalteu (12) was applied to the urine both before and after extraction with ether. Thus the total hydroxyphenyl compounds were divided into ether-soluble and ether-insoluble components.

#### RESULTS

##### *Daily excretion of metabolic products*

The results, calculated on the basis of 12-hour periods, are presented in Table I.

*Phenylpyruvic acid.* The patient always excreted phenylpyruvic acid in amounts varying from 0.45 to 1.03 grams per 24 hours. The gradual rise in excretion during the period of observation may perhaps have been due to his higher than customary intake of protein. In adult patients on a normal diet, Jervis (13) reported a daily output of 1.8 to 2.1 grams of phenylpyruvic acid, and Penrose and Quastel (11a), 1.4 grams.

*Phenyllactic acid.* The daily output of phenyllactic acid varied from 0.29 to 0.55 gram per 24 hours. Comparable figures for adult patients are not available.

*Phenylalanine.* The daily urinary output of phenylalanine varied from 0.21 to 0.32 gram. Because a search of the literature revealed no information on the normal urinary excretion of phenylalanine, a series of 11 24-hour urine specimens, from 5 children of comparable age and receiving similar diets, was analyzed. The results are presented in Table II, together with values

TABLE I  
Urinary excretion of protein derivatives in phenylpyruvic oligophrenia

Date	Weight	Nitrogen		Phenylalanine derivatives					Tyrosine derivatives		Substance ingested
		Intake	Urinary output	Phenyl-pyruvic acid	Phenyl-lactic acid	Phenylalanine			Ether-soluble expressed as tyrosine	Ether-insoluble expressed as tyrosine	
						Total	d-form	l-form			
	kgms.	mgm. per kgm. per 24 hours	mgm. per kgm. per 12 hours	mgm. per 12 hours					mgm. per 12 hours		grams
November 27 to 28	14.3		175 200	258 195	134 159	101 107	4 11	97 96	28 27	15 10	
November 28 to 29	14.6	672	200 180	260 271	245 265	124 139	19 5	105 134	28 47	20 14	
November 29 to 30	14.6	579	160 188	332 328	273 233	157 146	26 20	131 126	94 120	28 30	7.3 l-tyrosine
November 30 to December 1	15.0	640	200 250	454 461	276 273	168 155	29 23	139 132	78 105	38 57	
December 1 to 2	14.8	730	220 191	1988 1332	566 910	1951 686	1619 323	332 363	118 63	51 33	7.4 d,l-phenylalanine
December 2 to 3	14.8	640	274 219	1294 737	366 437	367 200	66 32	301 168	77 56	38 17	
December 3 to 4	14.8	592									
December 4 to 5	14.7	656	247 188	517 512	263 291	153 159	39 30	114 129	56 44	20 15	
December 5 to 6	14.6	656	209 142	508 356	233 152	179 91	35 24	144 67	52 32	17 11	0.2 ascorbic acid 0.2 ascorbic acid
December 6 to 7	14.9	640	193 198	446 447	199 200	163 141	18 13	145 128	37 34	13 14	0.2 ascorbic acid 0.2 ascorbic acid
December 7 to 8	14.8	683	184	2141	595	1388	844	544	54	14	0.2 ascorbic acid 7.45 d,l-phenylalanine
			205	1997	470	362	156	206	53	16	0.2 ascorbic acid
December 8 to 9	14.8	736	222 212	1069 666	327 240	261 190	35 9	226 181	19 25	53 32	0.2 ascorbic acid

obtained in previous studies in infants. In no instance did the phenylalanine excretion exceed 53 mgm. per 24 hours (4.52 mgm. phenylalanine nitrogen), the average being 30 mgm. per 24 hours (2.5 mgm. nitrogen) which is less than one-seventh the average amount excreted by our patient. In the normal children, the phenylalanine nitrogen amounted to 0.073 per cent of the total urine nitrogen whereas, in the patient with phenylpyruvic oligophrenia, the phenylalanine nitrogen represented 0.42 per cent of the total nitrogen. From this, it would appear that this patient, in contrast to the patient studied by Jervis (13), excreted phenylalanine in the urine in amounts significantly above normal.

The larger part of excreted phenylalanine was of the natural l-form. From 4 to 26 per cent (average 14 per cent) was d-phenylalanine. If this figure is to be considered valid in view of the technical difficulties inherent in the determinations of small amounts of d-phenylalanine, it must be

explained on the basis of racemization in the body, inasmuch as phenylalanine in the food is undoubtedly l-phenylalanine. Such *in vivo* racemization has been shown to occur in the case of d-phenylaminobutyric acid (du Vigneaud and Irish (14)). Racemization would be consistent with the theory of Jervis and his colleagues (7) that the excess phenylalanine in the blood of phenylpyruvic oligophrenics is formed from phenylpyruvic acid, although Fölling, Closs, and Gamnes (15) maintained that the excess phenylalanine found in the blood in their cases was all the levo form.

*Tyrosine derivatives.* The daily excretion of hydroxyphenyl compounds averaged 77 mgm. for the ether-soluble and 30 mgm. for the ether-insoluble portion. In the 5 normal subjects concerning whom other data are presented in Table II, an average figure for the ether-insoluble fraction was 27 mgm. There is, therefore, no evidence of any abnormal excretion of hydroxyphenyl compounds in this case of phenylpyruvic oligophrenia.

TABLE II

*Urinary excretion of phenylalanine and tyrosine by young infants and children compared to that of the patient with phenylpyruvic oligophrenia, per 24 hours*

Subject	Age	Weight	Protein intake	Total nitrogen in urine	Tyrosine* nitrogen	Phenylalanine† nitrogen	Phenylalanine as per cent of total nitrogen
		kgm.	grams per kgm.	grams	mgm.	mgm.	
D. S. Eczema	3½ years	13.0	6.8	8.43 6.68	3.48 2.89	4.52 3.28	0.054 0.049
P. M. Tuberculosis	2½ years	14.2	2.6	1.62 2.32 1.69	2.25 2.21 2.20	2.23 1.78 3.36	0.138 (Thymol)§ 0.077 0.199 (Thymol)
J. L. Tuberculosis, malnutrition	4 years	13.4	3.5 4.7	3.53 5.38	1.31 2.50	2.09 4.41	0.059 0.082
A. K. Dwarfism	3½ years	8.7	4.1 2.9	2.32 4.47	0.60 1.41	0.66 1.68	0.028 0.038
W. K. Mastoid, post-operative	5 years	17.5	4.4 4.0	5.77 4.71	2.34 1.75	2.31 1.64	0.040 0.035
Premature infants 24 days on 9 infants	8 to 25 days	1.82 to 3.14	6.0	0.96	1.93	2.25	0.230
Full term infants 3 days on 2 infants	17 to 21 days	3.53 to 3.88	4.5	1.66	0.88	2.55	0.150
S. W. Phenylpyruvic oligophrenia	2 years	14.5	4.2 4.1	5.68 5.78	4.10 2.32	23.29 25.06	0.41 0.43

\* One mgm. tyrosine nitrogen is equivalent to 12.93 mgm. of the amino acid.

† One mgm. phenylalanine nitrogen is equivalent to 11.79 mgm. of the amino acid.

§ Poor specimen—several enureses.

### *Effect of ingestion of tyrosine*

Seven and three-tenths grams l-tyrosine (0.5 gram per kgm.) was administered by mouth in a single dose without significant effect on the urinary excretion of phenylpyruvic acid or phenylalanine (Table I). The slight increase in tyrosine and its derivatives represented roughly 2 per cent of the amount ingested and is not indicative of any fault in the metabolism of tyrosine. This confirms the results obtained by Jervis (13).

### *Effect of ingestion of phenylalanine*

The ingestion of 7.4 grams d,l-phenylalanine (0.5 gram per kgm.) caused a marked increase in the urinary excretion of phenylpyruvic and phenyllactic acids and phenylalanine. In the first 24 hours, 2.404 grams phenylpyruvic acid, 0.926 gram phenyllactic acid, and 2.313 grams phenylalanine were excreted in excess of the foreperiod levels, thus accounting for approximately 76 per cent of

the ingested dose (Table III). Within 48 hours, the extra excretion had amounted to 98 per cent of the ingested dose.<sup>2</sup>

The maximum excretion of phenylpyruvic acid and phenylalanine was reached during the first 12 hours, whereas phenyllactic acid excretion reached its peak during the second 12 hours.

The magnitude of phenylpyruvic acid increase after phenylalanine is comparable to that observed by Penrose and Quastel (11a) who gave 3 grams of phenylalanine to phenylpyruvic oligophrenic adults and obtained an excretion of extra phenylpyruvic acid equivalent to the total phenylalanine fed.

<sup>2</sup> As was suggested in the description of methods, the values for phenyllactic acid may be too high, both in foreperiods and after amino acid ingestion. Even disregarding entirely this fraction, a minimum of 64 per cent of ingested phenylalanine is accounted for in the first 24 hours by the excretion of phenylalanine and phenylpyruvic acid, and 82 per cent in 48 hours.

TABLE III

*Urinary excretion of phenylalanine derivatives after ingestion of d,l-phenylalanine*

	Phenyl- pyruvic acid	Phenyl- lactic acid	Phenylalanine		Total
			d-form	l-form	
INGESTION OF 7.4 GRAMS d,l-PHENYLALANINE					
Foreperiod level—mgm.	458	275	26	136	
Extra excretion					
First 12 hours—mgm.	1530	291	1593	196	
Per cent of intake*	20.7	3.9	21.5	2.6	48.7
2nd 12 hours—mgm.	874	635	297	227	
Per cent of intake	11.8	8.7	4.0	3.1	27.6
3rd 12 hours—mgm.	836	91	40	165	
Per cent of intake	11.3	1.2	0.5	2.2	15.2
4th 12 hours—mgm.	279	162	6	32	
Per cent of intake	3.8	2.2	0.1	0.4	6.5
					98.0
INGESTION OF 7.45 GRAMS d,l-PHENYLALANINE†					
Foreperiod level—mgm.	447	200	16	137	
Extra excretion					
First 12 hours—mgm.	1694	395	828	407	
Per cent of intake	22.7	5.3	11.1	5.5	44.6
2nd 12 hours—mgm.	1550	270	140	69	
Per cent of intake	20.8	3.6	1.9	0.9	27.2
3rd 12 hours—mgm.	622	127	19	89	
Per cent of intake	8.3	1.7	0.3	1.2	11.5
4th 12 hours—mgm.	219	40	—7	44	
Per cent of intake	2.9	0.5	—0.1	0.6	3.9
					87.2

\* The molecular weights of these three compounds are so nearly identical that calculations of percentages or sums of percentages are the same whether reported "as phenylpyruvic acid," "as phenyllactic acid" or "as phenylalanine."

† Diet unchanged except for ingestion of 0.4 gram ascorbic acid daily.

The high excretion of phenylalanine, both before and particularly after the ingestion of this substance, was in contrast to the observation of Jervis (13) who found none at all in the urine of a phenylpyruvic oligophrenic, even after administering phenylalanine by mouth. Fölling, Closs, and Gamnes (15), using a qualitative method, reported that an increased excretion of phenylalanine followed the oral or intravenous administration of d-phenylalanine to their human subjects.

It is interesting to note that only about 6 per cent of the ingested d,l-phenylalanine was excreted as the l-isomer, whereas 13 to 25 per cent of it was excreted as d-phenylalanine. Presumably the body excreted more of the unnatural enantiomorph which is less readily metabolized.

### *Effect of ascorbic acid*

Because vitamin C has been shown to prevent or relieve a defect in the metabolism of tyrosine and phenylalanine exhibited by premature infants (16), it was tested in this case of phenylpyruvic oligophrenia (Tables I and III). It was completely without effect in relieving the spontaneous phenylketonuria and did not prevent the increased excretion of the metabolic products of phenylalanine after the administration of the amino acid. It is unlikely that the small differences in the rate and pattern of excretion of the derivatives in the two sets of observations following phenylalanine ingestion are attributable to ingested ascorbic acid. Jervis (17) found similar results in his patients.

### DISCUSSION

The path of normal metabolism of phenylalanine is still a matter of speculation. Presumably, it is first deaminized to form the -keto acid, phenylpyruvic acid, which may then be completely oxidized. There is, however, evidence that at least some phenylalanine may be converted in the body to tyrosine or its metabolic products, p-hydroxyphenylpyruvic and p-hydroxyphenyllactic acids (18). In phenylpyruvic oligophrenia, there is apparently no impairment in the metabolism of tyrosine. Even after the administration of tyrosine or phenylalanine there is no significant increase in urinary excretion of substances giving the Millon reaction. This finding, taken in conjunction with the fact that ingested phenylalanine can be accounted for almost quantitatively as excess phenylpyruvic and phenyllactic acids and phenylalanine, would indicate that phenylpyruvic oligophrenics do not metabolize phenylalanine by way of tyrosine. It is interesting to speculate whether tyrosine, which is not considered an essential amino acid in normal individuals, may be essential in these cases. If patients with phenylpyruvic oligophrenia are unable to form tyrosine from phenylalanine or its metabolic products, then

TABLE II

*Urinary excretion of phenylalanine and tyrosine by young infants and children compared to that of the patient with phenylpyruvic oligophrenia, per 24 hours*

Subject	Age	Weight	Protein intake	Total nitrogen in urine	Tyrosine* nitrogen	Phenylalanine† nitrogen	Phenylalanine as per cent of total nitrogen
		kgm.	grams per kgm.	grams	mgm.	mgm.	
D. S. Eczema	3½ years	13.0	6.8	8.43 6.68	3.48 2.89	4.52 3.28	0.054 0.049
P. M. Tuberculosis	2¼ years	14.2	2.6	1.62 2.32 1.69	2.25 2.21 2.20	2.23 1.78 3.36	0.138 (Thymol)§ 0.077 0.199 (Thymol)
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the ingested dose (Table III). Within 48 hours, the extra excretion had amounted to 98 per cent of the ingested dose.<sup>2</sup>

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#### DISCUSSION

The path of normal metabolism of phenylalanine is still a matter of speculation. Presumably, it is first deaminized to form the -keto acid, phenylpyruvic acid, which may then be completely oxidized. There is, however, evidence that at least some phenylalanine may be converted in the body to tyrosine or its metabolic products, p-hydroxyphenylpyruvic and p-hydroxyphenyllactic acids (18). In phenylpyruvic oligophrenia, there is apparently no impairment in the metabolism of tyrosine. Even after the administration of tyrosine or phenylalanine there is no significant increase in urinary excretion of substances giving the Millon reaction. This finding, taken in conjunction with the fact that ingested phenylalanine can be accounted for almost quantitatively as excess phenylpyruvic and phenyllactic acids and phenylalanine, would indicate that phenylpyruvic oligophrenics do not metabolize phenylalanine by way of tyrosine. It is interesting to speculate whether tyrosine, which is not considered an essential amino acid in normal individuals, may be essential in these cases. If patients with phenylpyruvic oligophrenia are unable to form tyrosine from phenylalanine or its metabolic products, then



they must be dependent on a dietary intake of tyrosine for their metabolic needs of this amino acid.

Undoubtedly, phenylalanine is deaminized to phenylpyruvic acid, but the mechanism and site of this deamination are still uncertain. If it takes place chiefly in the liver, one would expect to find phenylpyruvic acid in the blood in the process of transportation to the kidneys. Both Fölling, Closs, and Gamnes (15) and Jervis and his colleagues (7) were unable to find significant amounts of phenylpyruvic acid in the blood of their patients, but they did find abnormally large amounts of phenylalanine in the blood. Jervis concluded that the essential defect of the subjects is an inability to dispose of phenylalanine at a normal rate rather than a failure to break down phenylpyruvic acid, as had been previously assumed. The presence of phenylpyruvic acid in the urine he considered to be an incidental phenomenon, resulting from the deamination of a portion of the blood phenylalanine by kidney tissue.

The excretion by our subject, S. W., of large amounts of phenylalanine after ingestion of this substance may have been due to the size of the dose given (7.4 grams, 0.5 gram per kgm. of body weight), which was much greater than that which Jervis (13) administered to his patient. The amounts of phenylalanine in the urine of S. W. were of the same order of magnitude as those found in normal infants given comparable doses (18). The latter, however, never exhibited the most striking metabolic characteristic of the phenylpyruvic oligophrenic, namely, the excretion of significant amounts of phenylpyruvic acid, and they did, in the absence of vitamin C, show evidence of conversion of phenylalanine to tyrosine which was not observed in the oligophrenic subject.

The clinical value of the qualitative test for phenylpyruvic acid in the urine of infants suspected of mental retardation should be emphasized. The test should be applied especially in those retarded children who are blond in coloring, and whose mental deficiency is suspected on the basis of slowness in acquiring behavior patterns and yet seems to be belied by an attractive facial appearance. The presence of eczema, of a family his-

tory positive for mental deficiency, or of neurological manifestations characteristic of extrapyramidal tract involvement, should also lead to a suspicion of this disease, though their absence does not rule it out.

#### SUMMARY

1. Studies were carried out on the metabolism of phenylalanine and tyrosine in a patient with phenylpyruvic oligophrenia.

2. The patient always excreted phenylpyruvic acid in the urine in amounts varying from 0.45 to 1.03 grams per 24 hours. The daily excretion of phenyllactic acid varied from 0.29 to 0.55 gram and of phenylalanine from 0.21 to 0.32 gram per 24 hours.

3. The administration of d,l-phenylalanine (0.5 gram per kgm.) resulted in a prompt rise in the excretion of phenylpyruvic and phenyllactic acids and phenylalanine. Within 24 hours after feeding, 76 per cent of it had been excreted; 32 per cent as extra phenylpyruvic acid, 13 per cent as phenyllactic acid, and 31 per cent as extra phenylalanine, about three quarters of the latter being d-phenylalanine and the remainder the l-isomer. Within 48 hours, the extra excretion had amounted to 98 per cent of the ingested dose.

4. The administration of l-tyrosine in similar dosage (0.5 gram per kgm.) had no significant effect on the excretion of phenylpyruvic acid, nor was there any evidence of an abnormality in the metabolism of tyrosine.

5. The administration of large doses of ascorbic acid (0.4 gram per day) had no effect, either on the spontaneous excretion of phenylpyruvic acid or on the increased excretion of phenylpyruvic acid following the ingestion of ascorbic acid.

6. The absence in (11a) who gave 3 grams amounts of hydroxyphenylpyruvic acid increase following the ingestion of phenylpyruvic acid comparable to that observed in phenylpyruvic oligophrenic patients, spontaneously and excretion of extra phenylalanine, suggested to the total phenylalanine *in vivo* of phenylalanine to take place in the nor-

in patients with phenylpyruvic description of methods, the finding is consistent with may be too high, both in fore- Jervis and his coworkers id ingestion. Even disregard- the primary metabolic a minimum of 64 per cent of accounted for in the first 24 handle phenylalanine. phenylalanine and phenylpyruvic hours.

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# STUDIES ON EXPERIMENTAL AND CLINICAL HYPOCHLOREMIA IN MAN<sup>1</sup>

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## INTRODUCTION

In a recent study (1), it was demonstrated that severe hypochloremia and alkalosis could be induced gradually in the dog, by means of continued loss of chloride, without the development of a marked azotemia or detectable renal injury. Kerpel-Fronius (2), Kerpel-Fronius and Butler (3), Ambard (4), and Hiatt (5) had previously made similar observations in the dog and rabbit. The purpose of the present investigation was to determine whether or not the same situation could be induced in man.

## METHOD OF STUDY

### I. *Experimental hypochloremia*

Chloride deprivation was produced rather slowly by Wangensteen aspiration of the gastric contents, for varying lengths of time in 2 adult men (Cases M. S. and W. L.) with pyloric obstruction secondary to duodenal ulcer.

The patients received no food or fluid by mouth during the period of chloride withdrawal; 3000 to 4500 cc. of 5 per cent glucose in distilled water were administered intravenously each day. The serum chloride, carbon dioxide, pH, and blood urea nitrogen were measured frequently (6). Renal function in case M. S. was determined by the urea clearance test (7). Measurements were made also of the serum calcium (8), phosphorus (9), and potassium (10). The cell volume was determined frequently by hematocrit readings. The plasma proteins were determined by the Hanna-Campbell method (11), a modified macro-Kjeldahl technique being employed for the nitrogen analysis; 4 per cent boric acid was used to absorb the ammonia released. The blood sodium was measured by a modification of the Butler-Tuthill method (12); the phosphate of the serum was removed by ignition with ferric sulfate and the sodium determined on an aliquot from which the insoluble iron salt had been removed by centrifugation. The urinary sodium was determined in case M. S. by the same technique as that used for the serum sodium, modified as to the volume used; ferric salt was added in amounts sufficient to prevent interference by phosphates. The total

base was measured by a modification of the Stadie-Ross technique (13); the serum was ashed as for the sodium analysis; the ash was taken up in 45 cc. of water, the bases precipitated with benzidine hydrochloride, and the difference between the titration of the acidity of this filtrate and that of the original reagents used as a measure of the total base.

The patients' body weights were checked frequently. The fluid intake and output were recorded daily. The pH of the urine was determined with a Beckman pH meter; the chloride content of the urine was measured by the Van Slyke-Sendroy method (14); and the ammonia plus ammonium salts determined by the technique of Henriques and Sørensen (15). The volume, pH, and chloride content of the aspirated gastric secretions were measured similarly.

Aspiration of the stomach was discontinued after a severe but safely obtainable state of chloride depletion had been achieved. The body chloride was then replaced gradually in case M. S., over a period of 26 days, by the addition of increasing amounts of salt. The body chloride was replaced more rapidly in case W. L. (within 6 days) by the parenteral administration of saline solution.

### II. *Hypochloremia incidental to ulcer therapy*

The problem was studied further in 10 patients with alkalosis and chloropenia complicating the treatment of peptic ulcer. While two factors were operative in this latter group, *i.e.*, chloride loss and alkali ingestion, these cases are included because of the absence of nitrogen retention and because the evidence indicated that the major, indeed, if not the only, factor in the acid-base disturbance was the hypochloremia.

## RESULTS

### I. *Experimental hypochloremia*

*Case M. S. (Unit no. 204878 (Figures 1 and 2).* A 38-year-old salesman had taken alkalis intermittently for 5 years for the relief of symptoms from a stenosing duodenal ulcer. He had been vomiting for 5 days prior to hospitalization. Five per cent glucose in both normal saline and distilled water was administered intravenously during the first 4 days. Continuous Wangensteen aspiration of the gastric contents was begun on the 5th day and continued for 96 hours. The

<sup>1</sup> This study was supported in part by a grant from John Wyeth and Bro., Inc., Philadelphia, Pennsylvania.

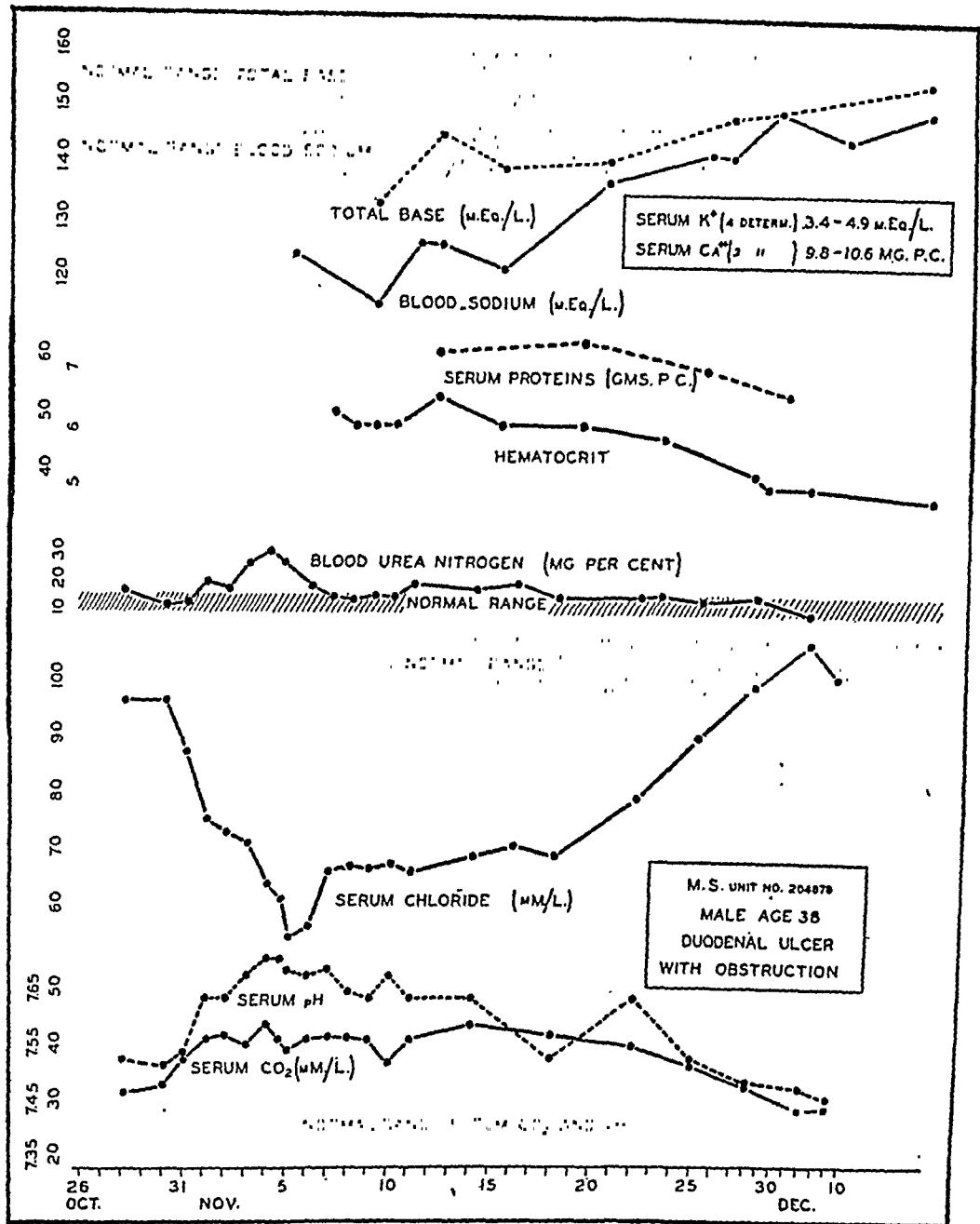


FIG. 1. EXPERIMENT 1. THE EFFECT OF A GRADUALLY INDUCED HYPOCHLOREMIA ON THE SERUM ELECTROLYTES IN MAN

gastric contents removed each day were acid, the pH ranging from 1.84 to 2.70; the amounts varied daily from 1100 to 3090 cc. Wangenstein suction was then limited to a period of 10 hours daily, the amounts withdrawn ranging from 440 to 1200 cc. The intravenous intake of fluid during the period of gastric aspiration consisted of 5 per cent glucose in distilled water, except for 1500 cc. normal saline given on the 12th day. This represented the only chloride (8.1 grams) taken by the patient during the interval from the 4th to the 16th hospital days. The salt intake was then increased very gradually until discharge from the

hospital on the 39th day, at which time the patient was receiving 3.7 grams of chloride daily. This amount was increased to 5.3 grams daily one week later, and the chloride intake was maintained at this level until the experiment had been concluded.

The lowest serum chloride of 53.8 mM./L. was obtained on the 11th day. The maximum blood urea nitrogen of 30 mgm. per cent was obtained at a time when the serum chloride measured 63.6 mM./L.; the values ranged usually from 14.2 to 19.6 mgm. per cent. The blood sodium decreased to 115.2 m.eq./L. and the total base to 133.3

m.eq./L. The serum calcium and potassium were normal. The urea clearance was not lowered significantly. The patient lost 23 pounds in weight; the presence of dehydration was further indicated by the increased hematocrit readings and the high plasma protein values.

The serum electrolytes, hematocrit, and plasma proteins gradually returned to normal during the period of chloride replacement. The blood urea nitrogen ranged from 8.1 to 14.3 mgm. per cent. At the conclusion of the experiment, the patient's body weight had returned to its original level. The daily volume of urine varied from 775 to 1920 cc., usually exceeding 1000 cc.; the pH ranged from 5.9 to 6.85. Its chloride content was markedly reduced; no chloride was found in the urine

on 5 days. Sodium excretion paralleled the values found for chloride ion. Ammonia excretion in the urine, as was to be expected, was diminished.

A histamine test performed on the 19th day (serum chloride between 65.5 and 68.8 mM./L.) revealed normal gastric secretion; the pH of the various samples of gastric juice ranged from 1.31 to 1.71. An examination of the spinal fluid on the 25th day (serum chloride between 68.5 and 78.8 mM./L.) revealed a clear fluid under normal pressure. The chloride measured 100.4 mM./L. and the total base 141.5 mM./L. (both values moderately reduced). The calcium was 4.8 mgm. per cent and the urea nitrogen 16.7 mgm. per cent (both values normal).

The patient was very weak and listless during

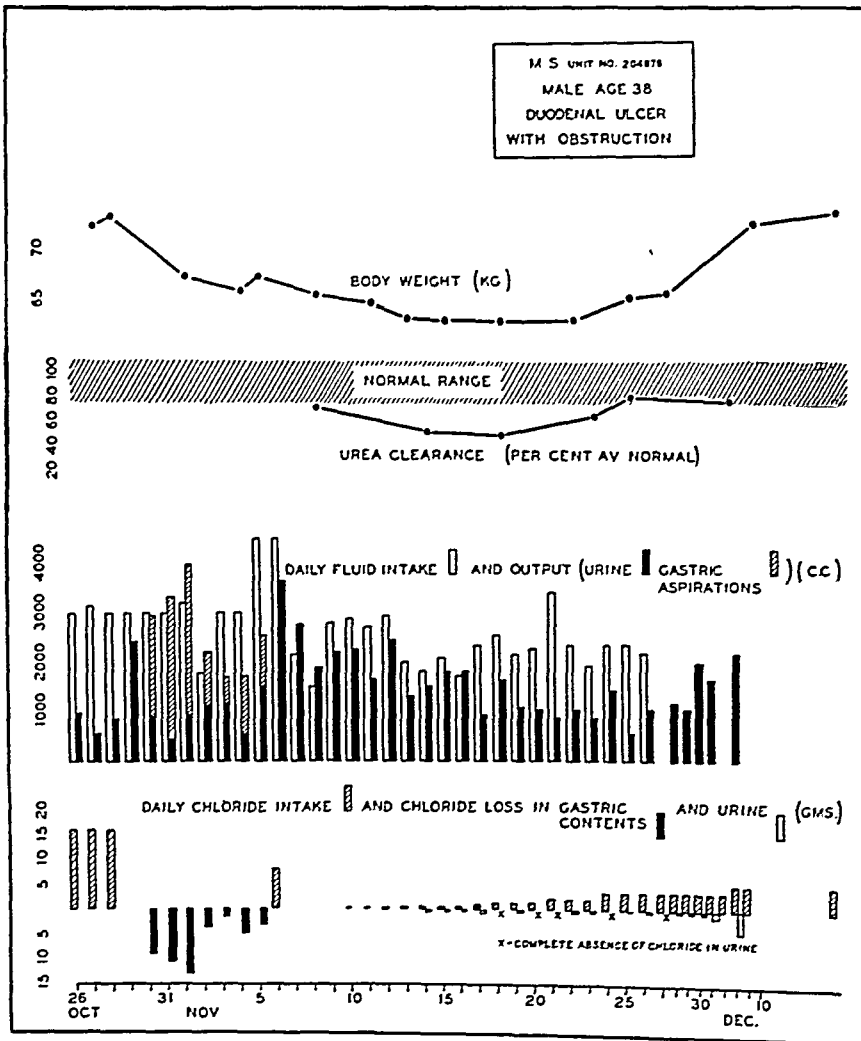


FIG. 2. EXPERIMENT 1. ADDITIONAL DATA. HYPOCHLOREMIA IN MAN

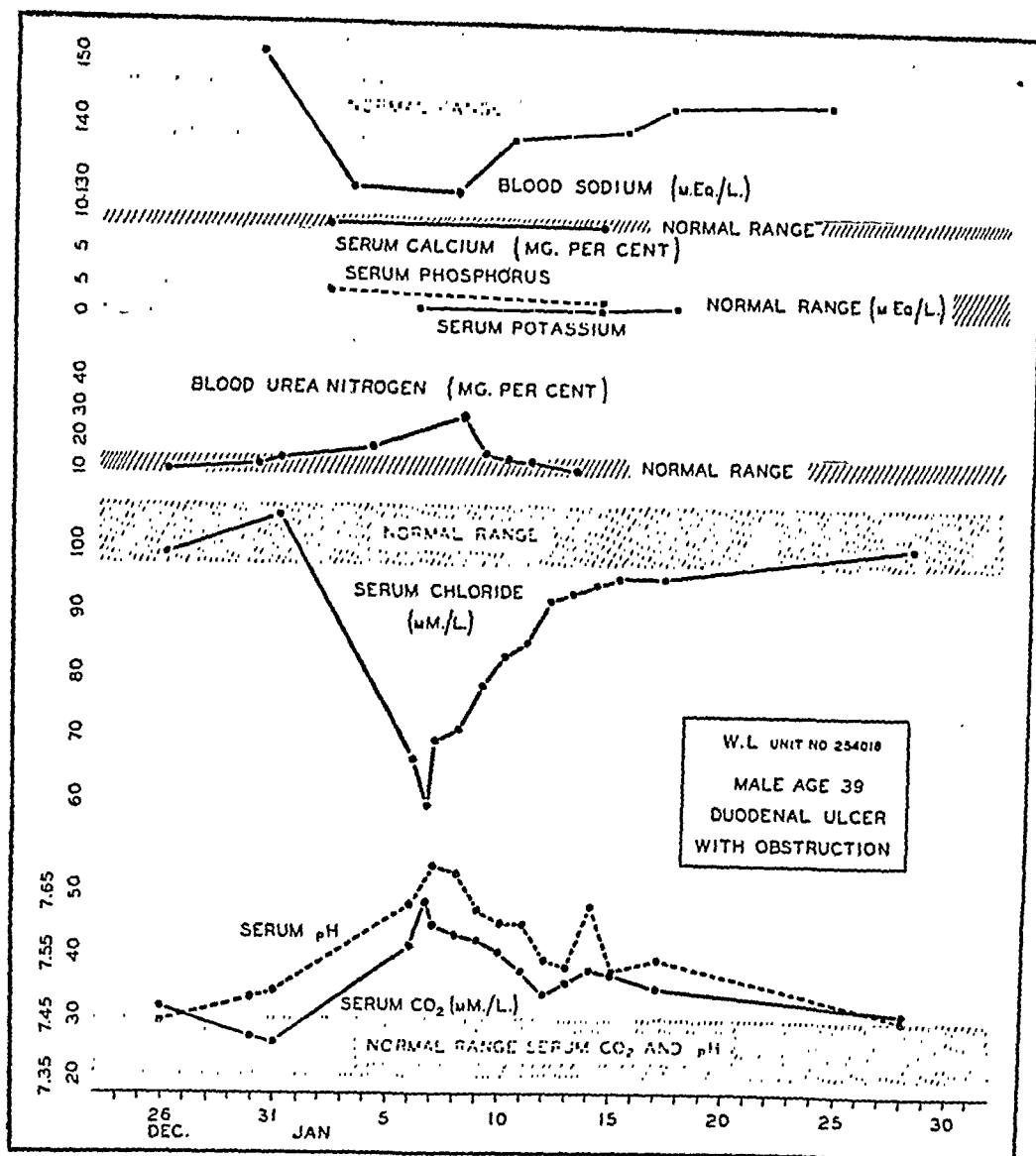


FIG. 3. EXPERIMENT 2. THE EFFECT OF A GRADUALLY INDUCED HYPOCHLOREMIA ON THE SERUM ELECTROLYTES IN MAN

the period of chloride depletion. He had no appetite and complained of a loss of the sense of taste. Mild muscular twitchings appeared on the 12th day (serum chloride 53.8 mM/L.) at which time the patient received the 1500 cc. normal saline intravenously, as noted above. The blood pressure, originally 130/95, decreased during the period of hypochloremia; the systolic values ranged from 90 to 110 and the diastolic from 60 to 72. The blood pressure at the completion of the experiment was 126/84. The patient's clinical course during the period of gradual chloride replacement was characterized by a steady gain in strength and improvement in appetite.

*Case W. L. (Unit no. 254018) (Figures 3 and 4).* A 39-year-old male had experienced ulcer symptoms for several years. He entered the hospital because of a recent massive hemorrhage and

marked pyloric obstruction. Therapy consisted of Wangensteen aspiration of the gastric contents, 3 blood transfusions, and, for the first week, 1500 to 3000 cc. of 5 per cent glucose in normal saline each day. On the 8th day and for the next 5 days, the patient received 5 per cent glucose in distilled water. An average of 1860 cc. of gastric contents were aspirated daily; the pH of the aspirates ranged from 1.94 to 2.58. Wangensteen suction then was discontinued; the patient, however, vomited large amounts of gastric contents on several occasions. Saline therapy was resumed on the 14th hospital day and the patient was given from 3000 to 4500 cc. of 5 per cent glucose in normal saline and distilled water daily. Aspiration of the stomach was resumed on the 20th day but the electrolyte loss now was replaced by the use of parenteral fluids.

The serum chloride decreased to 58.9 mM./L. The maximum blood urea nitrogen was 23.1 mgm. per cent, with the values ranging usually from 13.1 to 21.2 mgm. per cent. The lowest blood sodium was 129.2 m.eq./L., obtained on the day of the lowest serum chloride. The serum calcium, phosphorus, and potassium were normal. Adequate urea clearance studies were not obtained in this case. The patient lost 8 pounds in weight. The plasma protein and hematocrit values were elevated. The volume of urine varied from 660 to 4000 cc. daily, usually approximating 3000 cc.;

the pH ranged from 6.41 to 7.48. The chloride content of the urine was markedly reduced.

The patient gradually became very weak and listless during the period of chloride depletion. His blood pressure, originally 125/92, decreased slightly; the systolic ranged from 90 to 116 and the diastolic, from 68 to 90. These manifestations disappeared rapidly, however, as the acid-base balance was restored.

*Comment.* The maximum blood urea nitrogen values of 30.0 and 23.3 mgm. per cent noted in these 2 cases are in sharp contrast to the severe

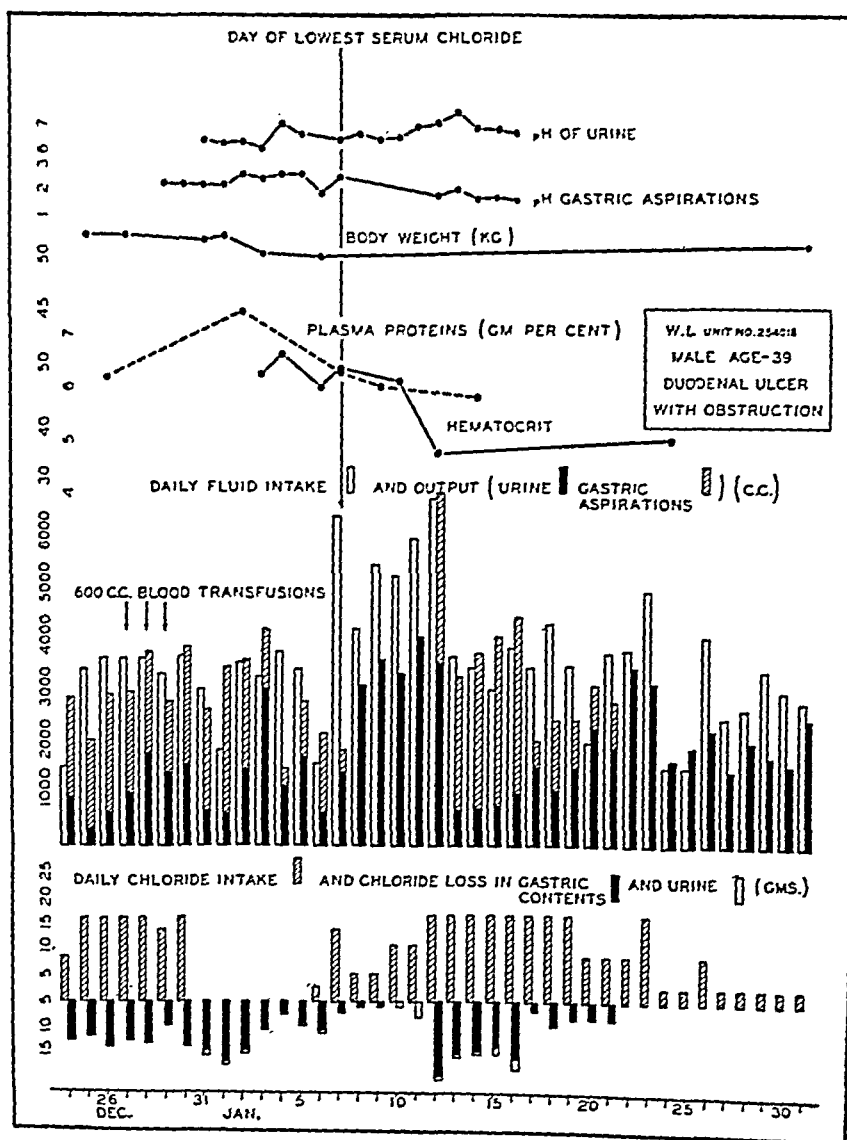


FIG. 4. EXPERIMENT 2. ADDITIONAL DATA. HYPOCHLOREMIA IN MAN.



nitrogen retention observed by other workers in patients with hypochloremia and alkalosis secondary to pyloric obstruction, or during experimental salt deficiency produced by diet and sweating (16), but are in agreement with the findings

of Kerpel-Fronius (2) and Kerpel-Fronius and Butler (3). Dehydration was present in both patients as evidenced by the significant weight loss and the high hematocrit and plasma protein values. Since large quantities of glucose in distilled

TABLE I  
*Hypochloremia without azotemia during alkalosis*  
(10 Patients)

Case Sex—Age	Acid-base balance			BuN	Urea clearance	Day of treatment	Gastric aspiration		Therapy
	Serum Cl	Serum CO <sub>2</sub>	pH				Average daily amount	Num- ber of days	
	mM./L.	mM./L.		mgm. per cent	per cent average normal		cc.		
J. S. 264531 M—26	65.9 92.1 92.2	38.0 32.0 35.1	7.52 7.47 7.56	18.4 16.9	90 110	2nd 3rd 5th	622	4	Calcium carbonate; parenteral fluids; gastroenterostomy on 8th day.
J. C. 185870 M—29	79.0 67.6 85.2	38.6 38.2 32.4	7.62 7.63 7.50	21.2 14.6 8.8	113 200 91	4th 7th	393	11	Sippy powders; NaCl (4 to 6 grams) started on 6th day.
D. H. 112315 M—45	98.4 80.4 92.8	26.3 36.9 35.2	7.47 7.62 7.47	10.4 7.9	85	3rd 11th 15th	217	16	Calcium carbonate; NaCl (5 grams) started on 14th day.
J. K. 198489 F—55	90.0 86.2 89.2	29.9 35.0 34.9	7.39 7.46 7.50	15.0 9.1 13.7	41 67 53	4th 12th 16th	207	20	Calcium carbonate; no NaCl.
T. S. 212524 M—49	94.8 81.8 84.8 97.6 99.0	32.8 36.4 31.5 28.8	7.48 7.54 7.49 7.47 7.48	14.5 19.6 15.2 10.4 8.1		6th 12th 18th 29th 33rd	148	16	Calcium carbonate; no NaCl.
F. W. 194370 M—43	96.7 82.8 82.6 98.8 96.6	31.7 37.3 37.5 29.5 31.2	7.56 7.55 7.53 7.38 7.44	12.9 12.4 12.2 11.4	143 93 200	2nd 9th 14th 18th 22nd	177	17	Sippy powders NH <sub>4</sub> Cl (4 to 6 grams) 15th to 22nd days.
W. B. 211777 M—51	80.6 85.6 98.1 96.8 97.2	31.8 47.4 41.1 28.0 32.4 34.2	7.48 7.73 7.62 7.49 7.49 7.45	18.7 20.6 18.7 8.8 16.9 18.3 15.3	96 128 54 69 148	7th 13th 16th 28th 40th 47th 1 year later	354	43	Calcium carbonate; NaCl (10 grams) 8th to 22nd days; various alkalis from 25th to 38th days; calcium carbonate then resumed.
H. B. 225751 M—49	90.4 83.0 97.0 101.8	30.6 39.4 35.0 31.0	7.50 7.62 7.47 7.47	23.2 18.7 15.7 11.7 12.0	85 76 102	4th 9th 13th 17th 4 months later	356	17	Calcium carbonate; NaCl started on 10th day; 200 cc. 5 per cent saline intravenously on 10th day.
S. S. 101209 M—37	84.3 95.2 100.2	42.9 30.9 36.0 32.9	7.71 7.51 7.53 7.50	17.3 15.0 11.1	83 112	15th 18th 22nd 24th 1 week later	85	20	Sippy powders; NH <sub>4</sub> Cl (2 to 6 grams) from 16th to 20th days.
A. G. 232838 M—52	96.0 89.4 87.8 87.4 91.9	31.1 34.4 35.6 36.7 35.1	7.54 7.47 7.48 7.49 7.52	11.7 22.4 14.2 11.7	80	2nd 4th 8th 10th 13th	100	15	Calcium carbonate; NaCl (3 grams) started on 12th day.

water were administered daily, it would appear that, as in the dog experiments, dehydration did not exist in the sense of deprivation of water but was present in the sense that fluid could not be retained in the body due to the marked depletion of electrolytes. The volume of urine excreted by both patients during the period of hypochloremia was large; the daily output averaged 1340 cc. in case M. S., and 1317 cc. in case W. L. The urea clearance in case M. S. was not altered significantly, and the absence of a marked elevation of the blood urea nitrogen is further evidence of satisfactory renal function. Despite the severe chloropenia, the pH and chloride content of the gastric aspirations were reduced only slightly; and the pH values of the histamine-stimulated gastric juice in case M. S. were as low as one finds in the histamine-stimulated gastric secretion of individuals with a normal acid-base balance. This finding is in accord with the observations made in dogs (1). The decrease in the spinal fluid chloride and total base noted is, of course, to be expected; McCance (17) and Agar and MacPherson (18) have reported similar findings. It is of interest that these values were not lower than they were, in view of the severe hypochloremia and hyponatremia. The explanation for this discrepancy is not apparent.

## II. Hypochloremia incidental to ulcer therapy

In Table I are recorded the acid-base balance and other data in 10 patients with alkalosis and hypochloremia, complicating the antacid treatment of peptic ulcer. Large amounts of gastric content had been aspirated in 8 of this group. In no instance was there any significant azotemia. Hypochloremia apparently had occurred gradually in all 10 patients.

## GENERAL DISCUSSION

This study confirms the results of the animal experiments referred to earlier, as well as the work of Kerpel-Fronius, and Kerpel-Fronius and Butler, and demonstrates that, in man also, a severe hypochloremia is not necessarily accompanied by azotemia. The important factors which determine the extent of urea nitrogen elevation during hypochloremia, apparently, are the speed with which

the chloride loss is induced, and the degree and rapidity of the dehydration associated with the chloropenia. An abrupt depletion of chloride is associated with an equally abrupt and marked loss of sodium and, therefore, of body water, as shown by Gamble and others (19). Loss of fluid decreases the circulating blood volume which, by reducing the venous return to the heart, presumably lowers the cardiac output. This course of events may actually lead to peripheral circulatory failure. The blood flow through the kidney and the effective glomerular filtration pressure are consequently lowered. The severe nitrogen retention and decreased renal function, noted under these conditions by other workers, evidently represent a decreased renal circulation rather than intrinsic renal disease. The absence of azotemia during hypochloremia in the present experiments may be attributed to two factors: (a) the gradual deprivation of chloride, allowing the patients to make fairly satisfactory adjustments to the severe electrolyte changes, and (b) the daily administration of large quantities of water which tended to "wash out" the urea nitrogen and apparently maintained an adequate blood flow through the kidney.

## CONCLUSIONS

1. Severe alkalosis without marked nitrogen retention may be induced in man by the gradual withdrawal of gastric secretion.
2. Such hypochloremia and alkalosis are not associated with decreased renal function, as measured by the urea clearance test, when adequate quantities of fluid are administered daily.
3. Gastric secretion in man is not altered significantly by severe hypochloremia.

The authors wish to acknowledge the valuable technical assistance of Miss Jacqueline Front.

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# GASTRIC SECRETION. II. ABSORPTION OF RADIOACTIVE SODIUM FROM POUCHES OF THE BODY AND ANTRUM OF THE STOMACH OF THE DOG

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The biochemical process by which cells of the stomach produce hydrochloric acid remains a mystery. Although the approximate concentration of the acid has been calculated on indirect evidence, the exact concentration as it is secreted has not been determined in any animal. This is despite the fact that, over a period of many years, increasingly precise observations have been made.

The difficulty confronting observers is that the same area of the stomach which secretes the hydrochloric acid also forms pepsin and mucin, and allows the passage of certain inorganic electrolytes and urea between the blood and stomach lumen. Little light has been thrown on this subject by analyses of the gastric contents of the stomach, since swallowed saliva and regurgitated secretions from the duodenum inconstantly contaminate the contents. Gastric pouches were first conceived to discount the inaccuracies of studying gastric contents, and indeed, examination of fluid collected from a pouch of the stomach of an experimental animal yields accurate information. It is frequently assumed, however, that this fluid from pouches represents true secretion. No account is taken of a possible concomitant absorption of water or electrolytes from the gastric lumen into the lymphatics or blood stream. Unless reabsorption can be excluded, the fluid within a pouch does not represent secretion but the resultant of secretion and absorption.

During the course of experiments on the effect of the osmotic pressure of gastric pouch contents on the volume of secretion (1), it became obvious that absorption from the pouch must be controlled. It is known that some substances are absorbed from the stomach in small quantities (2), but it is not known whether all of the observed absorption takes place from the antrum or from the body of the stomach. In contrast to the body of the stomach which secretes the hydrochloric acid, the

antrum secretes a neutral mucoid liquid. Since the main part of absorption from the gastrointestinal tract takes place in the intestine, the contents of which are neutral or alkaline, it is considered possible that most or all of the absorption attributed to the stomach might well occur in the antrum.

Recently, Eisenman *et al.* (3) have reported the absorption of various radioactive isotopes from the stomach, but no attempt to differentiate between antrum and body was reported.

The experiments reported in this and the subsequent paper (4) were devised, not only to examine the conditions controlling absorption from the stomach, but also to judge the relative absorptive powers of the antrum and body of the stomach.

## METHODS

Dogs with a pouch of either the body or antrum of the stomach were used. Pouches of the body were made in the manner previously described (5). There is no leakage from these pouches. Fluid can be introduced or withdrawn from the pouch by passing a catheter through the stoma in the flank.

In order to maintain the chloride balance, ammonium and sodium chloride are given by stomach tube to the dogs at regular intervals throughout the day. One thousand to 1500 cc. of acid secretion are lost daily from the pouch. This amounts to nearly one-third of the available fluid volume of the dog. Since the chloride concentration of the secretion is higher than that of plasma or lymph, it is obvious that such animals would be rapidly depleted of chloride if this electrolyte were not returned constantly. The gastric fluid collected from the pouch is not returned to the animal because it is often alkaline from an excess of sodium bicarbonate solution, put in after the previous emptying. Neutralization of the gastric secretion is necessary over any extended period to prevent peptic digestion of the pouch wall. Bicarbonate solution is omitted the afternoon before an experiment.

Pouches of the antrum were made by a two stage technique. At a first operation, the antrum was divided from the body of the stomach. Both the anterior and posterior walls of the stomach were divided from the incisura to

the opposite point on the greater curvature. The terminal branches of both vagus nerves which pass to the antrum (5), and the sympathetic nerves which pass with the vessels, were spared. The open end of the antrum was closed to form the antral pouch. The open end of the body was anastomosed to the upper jejunum. At a second operation, 3 or more weeks later, when the suture lines of the first were well healed, the duodenum was divided 0.5 cm. distal to the pyloric ring. The duodenal

stump was turned in. The pylorus with the cuff of duodenum was then pulled through a stab wound in the right flank just below the costal margin, forming the stoma of the pouch. The only difficulty encountered in the operative procedures was in obtaining sufficient mobility of the antrum without dividing nerves and vessels. The nerve supply to the pylorus was sacrificed.

The secretion from such a pouch is thickly mucoid; its volume is small and grossly not increased during diges-

TABLE I

*The concentration of Na<sup>24</sup> in the blood serum at intervals after its introduction into the pouches of dogs with body pouches, and the chemical determinations on gastric contents and blood serum*

Where two sets of figures are given for both gastric contents and blood serum, the top figure represents the determination on contents and blood removed immediately prior to the introduction of the Na<sup>24</sup>Cl solution; the bottom figure (in italics) represents the determination made on the contents and blood removed at the end of the experiment. Where only one figure is given for the blood, a single blood sample was removed in the middle of the experiment. Dog number 1: weight 21.7 kgm., plasma volume 854 cc., interstitial fluid volume 4538 cc., available fluid 5621 cc. Dog number 2: weight 21.8 kgm., plasma volume 1064 cc., interstitial fluid volume 4891 cc., available fluid 5955 cc. Dog number 3: weight 19.4 kgm., plasma volume 796 cc., interstitial fluid 5190 cc., available fluid 6178 cc.

Experiment number	NaCl concentration State of pouch	Na <sup>24</sup>				Gastric content			Blood serum				Date
		Blood serum				pH	Cl	Total base	Cl	CO <sub>2</sub>	Total base	Na	
		15'	30'	60'	120'								
Dog 1		p.p.m. per cc.					m.eq. per L.		m.eq. per L.				
1	0.9 per cent Secreting	0.5	1.2	2.1									December 18, 1940
2	Secreting	1.5	5.0	2.8		1.3 1.4	130 144		112 116		157 159	152 152	February 5, 1941
3	Secreting			0.6	3.2	1.0 1.2	154 156		100 100	32 32	155 155	145 145	June 5, 1941
4	Secreting			3.1	3.6	6.9 1.3	144 156	140 61	68	59	146	138	October 10, 1941
5	Fasting	0.9	2.3	4.5									January 22, 1941
6	Fasting	2.2	5.0	9.6		8.2	147	143	82 84	45 46	144 145	144 149	February 27, 1941
7	Fasting	2.0	3.6	8.4	20.5	2.1	137	102	104	32	157	153	March 26, 1941
8	Fasting			7.4	11.5	2.0 5.5	152 158	135 156	108 108	33	152 152	140	October 1, 1941
9	3.1 per cent Secreting			1.1	1.9	1.0 1.3	157 206		104 108	33 33	154 157	145	June 12, 1941
10	Secreting			1.1	1.5	0.9 1.1	160 208		102	31	155	144	June 24, 1941
11	Secreting			2.6	2.4	1.1 1.3	156 202		110	26	157	144	July 8, 1941
12	0.4 per cent Secreting			2.9	7.3	0.9 1.0	157 150		111	27	154	145	July 17, 1941
13	Secreting			1.9	5.3	1.0 1.1	154 147		108	28	155	143	July 25, 1941

TABLE I—Continued

Experiment number	NaCl concentration State of pouch	Na <sup>24</sup>				Gastric content			Blood serum				Date
		Blood serum				pH	Cl	Total base	Cl	CO <sub>2</sub>	Total base	Na	
		15'	30'	60'	120'								
Dog 2		p.p.m. per cc.				m.eq. per L.			m.eq. per L.				
14	0.9 per cent Secreting	0.3	0.4	0.7									January 22, 1941
15	Secreting	1.4	1.6	2.8		1.5	160	114	106 106	32 35	154 158	166 167	February 27, 1941
16	Secreting			1.6	2.0	1.3 1.3	140 158		106 102	25 31	152 168	147	June 5, 1941
17	Fasting	3.3	7.6	13.2									December 18, 1940
18	Fasting	4.0	6.5	10.0		7.0 7.6	128 160		90 94		148 148	140 144	February 5, 1941
19	Fasting	34.0	58.0	76.0	102.0	7.2	116	163	92	38	154	150	March 26, 1941
20	3.1 per cent Secreting			2.8	4.2	1.1 1.4	144 218		106 108	29 29	157 157	145 146	June 12, 1941
Dog 3													
21	3.1 per cent Secreting			3.5	3.5	1.1 1.1	147 204		112	25	161	151	June 24, 1941
22	Secreting			2.6	3.0	1.1 1.4	145 215		104	27	150	139	July 8, 1941

tion of food in the body of the stomach. The pH of the secretion is usually around 7 but in some animals may fall during active gastric digestion. In this case, there are a few parietal cells presumably included in the pouch. The pouch has strong muscular contractions and rarely contains more than 1 to 2 cc. In a large dog, the pouch may be made so it will contain 15 cc. The experiments were run with a catheter in place and an open syringe attached to the outer end. The fluid level in the syringe was kept a few centimeters above the pouch. The test fluid fluctuated with each period of relaxation and contraction of the pouch. With severe contractions, leakage sometimes occurred around the catheter at the stoma.

Radioactive sodium (Na<sup>24</sup>) was prepared by bombardment of metallic sodium with deuterons.<sup>1</sup> This was converted into NaCl and made up to the desired concentration with water. Measurements of radioactivity were made with the aid of a Geiger-Muller counting rate meter and a Lauritzen electroscope. All results are corrected for radioactive decay, and are expressed in arbitrary units. One unit represents one-millionth part of the ad-

ministered Na<sup>24</sup> and is represented by the symbol p.p.m. (parts per million).

In order to bathe the mucous membrane of the pouch evenly, 30 cc. of the solution containing the radioactive sodium were placed in the pouches of the body. In the antral pouches, 10 to 15 cc. were used.

The absorption of radioactive sodium was determined from the concentration observed in the blood stream. Because of the small amount of sodium actually absorbed, and some leakage in the case of the antral pouches, the estimation of the total sodium absorbed, determined by subtracting the sodium recovered in the pouch at the end of the experiment from the sodium put in at the beginning, is inaccurate. The determination of the radioactive sodium within the blood serum gave reproducible results with an error of less than 5 per cent. From these serum concentrations, and the combined plasma and interstitial fluid volumes as determined with Evans blue and thiocyanate (6), the total Na<sup>24</sup> absorbed was calculated on the assumption that it was evenly distributed extracellularly. The small amount in the erythrocytes (7) and bone (8) is negligible.

All of the chemical determinations have been made by standard laboratory methods. All blood samples on which electrolytes were determined were withdrawn from a femoral artery.

<sup>1</sup> We are indebted to Dr. B. Curtis and the crew of the Harvard cyclotron for the radioactive material used in these experiments.

EXPERIMENTS

First, the state of the dog, secreting or fasting, was established. In those experiments where the animals were secreting, that is, in an active state of gastric digestion, food had been given by mouth at 8:00 a.m. In the dogs with pouches of the body of the stomach, the output into the pouch was measured hourly for the ensuing 2 to 4 hours. A flow of juice of low pH and increased volume, comparable to control days for that dog after feeding, was considered adequate proof of active secretion. In the dogs with an antral pouch, the only evidence obtainable of active gastric digestion was a drop in pH of the secretion.

In the experiments with animals in the fasting state, the last feeding had been given 24 to 48 hours previous to the day of experiment. Close control of the fasting state was necessary because of the occasional reappearance of active secretion, due presumably to a conditioned reflex. In the animals with the body pouch, a low volume output of juice of high pH was demonstrated for at least 2 hours.

Second, arterial blood was taken for control determinations of electrolytes. (This initial control was omitted in some of the later experiments.)

Third, the radioactive sodium, in isotonic, hypertonic, or hypotonic solution was introduced into the pouch. Fourth, blood was removed at intervals up to 2 hours for determination of radioactivity of the serum. On the last or middle sample, the total electrolyte partition was determined. Finally, the pouch was emptied, and the volume, pH, pepsin, and electrolytes of the contents determined. The pouch was rinsed twice and the total recovery of radioactive sodium measured from contents and rinsings.

Three dogs with a pouch of the body of the stomach, and 2 with a pouch of the antrum, were used. The experiments were grouped as follows:

1. Body pouch—Secreting state—Isotonic solution of radioactive sodium

Seven experiments with an isotonic solution of  $\text{Na}^{24}\text{Cl}$  were performed on 2 of the dogs with a pouch of the

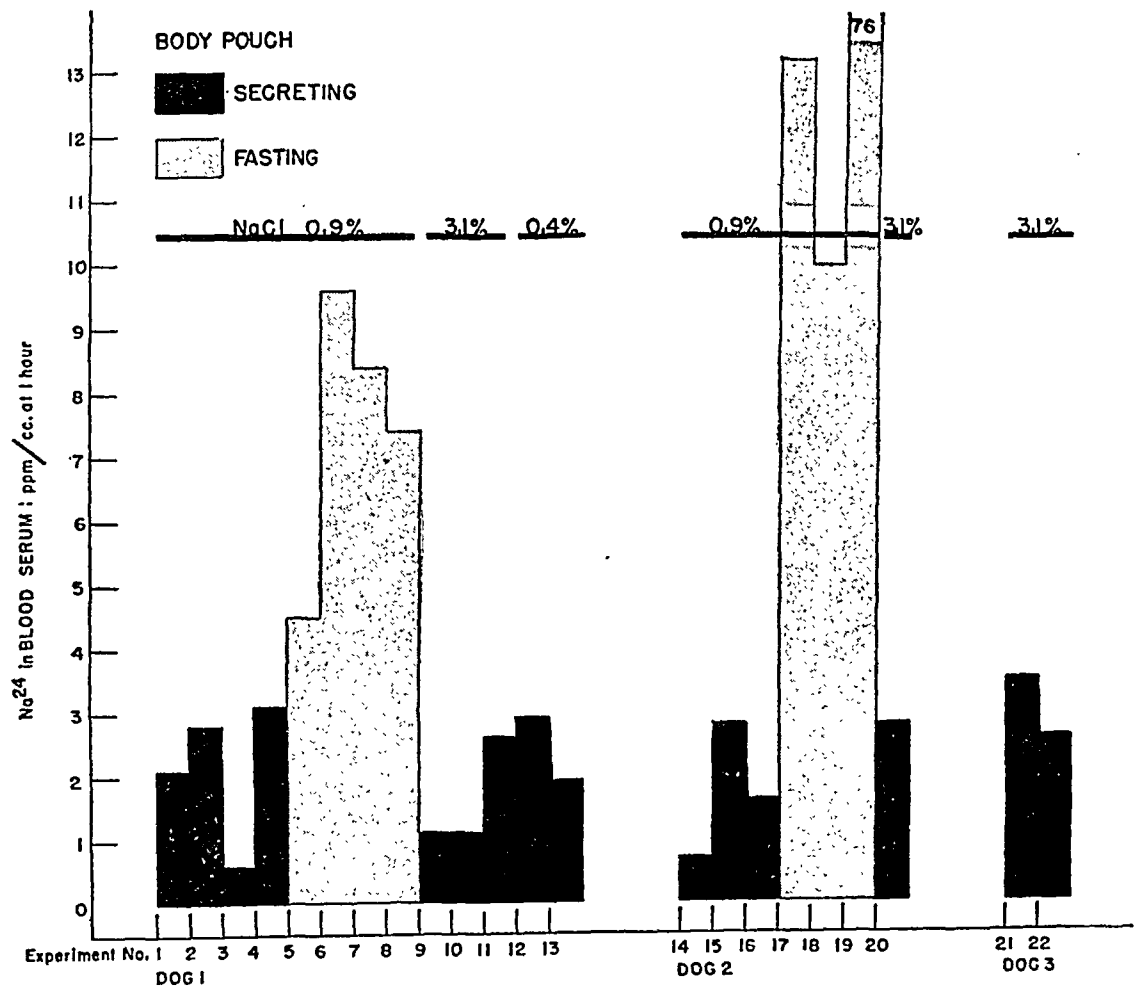


FIG. 1. THE CONCENTRATION OF  $\text{Na}^{24}$  IN THE BLOOD SERUM ONE HOUR AFTER ITS INTRODUCTION INTO THE POUCHES OF THE 3 DOGS WITH POUCHES OF THE BODY OF THE STOMACH

In the experiments represented by the black blocks, the pouches were actively secreting hydrochloric acid; in those represented by the gray blocks, the dogs were fasting and the pouches were secreting a neutral to slightly acid juice. The percentage of the  $\text{Na}^{24}\text{Cl}$  solution used in the various experiments is given at the upper half of the chart.

body, during the secreting state. The results of the determinations on gastric juice and blood are given in Table I, experiment numbers 1 through 4, and 14 through 16. The concentration of radioactive sodium in the blood serum, one hour after the introduction of the sodium into the pouch, is shown graphically in Figure 1.

An appreciable concentration of radioactive sodium was found in the blood stream, 15 minutes after the introduc-

tion of the sodium solution into the pouch. The concentration rose through the 1 or 2 hours of each experiment. The absolute amounts of radioactive sodium absorbed by the 2 dogs were approximately the same.

In order to determine if variations in electrolyte concentrations in the blood would exert any effect on the rate of absorption of sodium, the intake of chloride and sodium by mouth was altered during the days previous to the

TABLE II

*The concentration of Na<sup>24</sup> in the blood serum at one and 2 hours after its introduction into the pouches of the 2 dogs with antral pouches, and the chemical determinations on gastric contents and blood serum*

Where two sets of figures are given for both gastric contents and blood serum, the top figure represents the determination on contents and blood removed immediately prior to the introduction of the Na<sup>24</sup>Cl solution; the bottom figure (in italics) represents the determination made on the contents and blood removed at the end of the experiment. Where only one figure is given for the blood, a single blood sample was removed in the middle of the experiment. Dog number 4: weight 17.3 kgm.; dog number 5: weight 18.5 kgm.

Experiment number	NaCl concentration State of pouch	Na <sup>24</sup>		Gastric content	Blood serum				Date
		Blood serum		pH	Cl	CO <sub>2</sub>	Total base	Na	
		60'	120'						
Dog 4		p.p.m. per cc.			mg. per L.				
23	0.9 per cent Secreting	9.0	21.2		114 112	20 23	156 161	148 149	June 5, 1941
24	Secreting	2.0	2.8		108	29	151	145	October 10, 1941
25	Fasting	14.1	14.9		117	23	149	141	October 1, 1941
26	3.1 per cent Secreting	12.5	16.0	2.5	112	23	152	144	June 24, 1941
27	Fasting	17.5	31.4		120 114	19 21	155 162	144	June 12, 1941
28	Fasting	15.5	23.3	6.6 4.5	112	23	157	144	July 8, 1941
29	0.4 per cent Secreting	3.6	4.1	2.7 2.0	110	28	152	143	July 17, 1941
30	Fasting	7.5	8.8	2.5	110	24	151	143	July 24, 1941
Dog 5									
31	0.9 per cent Secreting	19.2	58.5	9.0 7.8	118	27	155	148	October 10, 1941
32	Fasting	28.0	47.0		110 114	26 26	153 155	144 148	June 5, 1941
33	3.1 per cent Secreting	55.0	68.5		106 106	32 32	162 160	150 149	June 12, 1941
34	Secreting	102.0	95.0	6.8	104	32	157	143	July 8, 1941
35	Fasting	83.0	68.0	7.4	96	31	153	140	June 24, 1941
36	0.4 per cent Secreting	42.7	54.5	3.2	108	31	161	149	July 25, 1941
37	Fasting	62.5	32.6	5.5	114	24	155	147	July 17, 1941



several experiments. Since these dogs lose daily such large quantities of electrolytes, principally chloride but also some base, through the secretion into the pouch, it is easy to produce rapid changes in electrolyte concentrations in the body fluids. In these experiments, a range of blood chloride from 116 m.eq. to 68 m.eq., and total base from 168 m.eq. to 146 m.eq., was obtained. No significant difference in the absorption of  $\text{Na}^{24}$  was observed in spite of these wide variations.

2. Body pouch—Fasting state—Isotonic  $\text{Na}^{24}\text{Cl}$  solution

Seven experiments with isotonic  $\text{Na}^{24}\text{Cl}$  solution were performed with 2 dogs in the fasting state (the same dogs used in the first group of experiments). The results of the determinations on gastric juice and blood are given in Table I, experiment numbers 5 through 8, and 17 through 19. The concentration of radioactive sodium in the blood serum, one hour after the introduction of the sodium into the pouch, is shown graphically in Figure 1.

With the mucous membrane of the pouch in the resting phase, there is a significant increase in the concentrations of the radioactive sodium in the blood serum over those found when the membrane is actively secreting hydrochloric acid. In dog 1, the average concentration in the serum at one hour was 7.5 p.p.m. in the resting state, against 2.2 p.p.m. when secreting; in dog 2, the average concentration at one hour was 33.2 p.p.m. resting, against 1.7 p.p.m. secreting. In the latter animal, the fasting average was weighted by one observation considerably higher than the other two. This single observation was the highest concentration in the serum recorded in any of the experiments with a pouch of the body of the stomach.

As in the experiments in group 1, shifts in electrolyte partition of the blood serum were obtained by altering the salt intake. The chloride observations varied from 108 to 82 m.eq., the total base from 157 to 144 m.eq. No significant correlation was determined between any of the

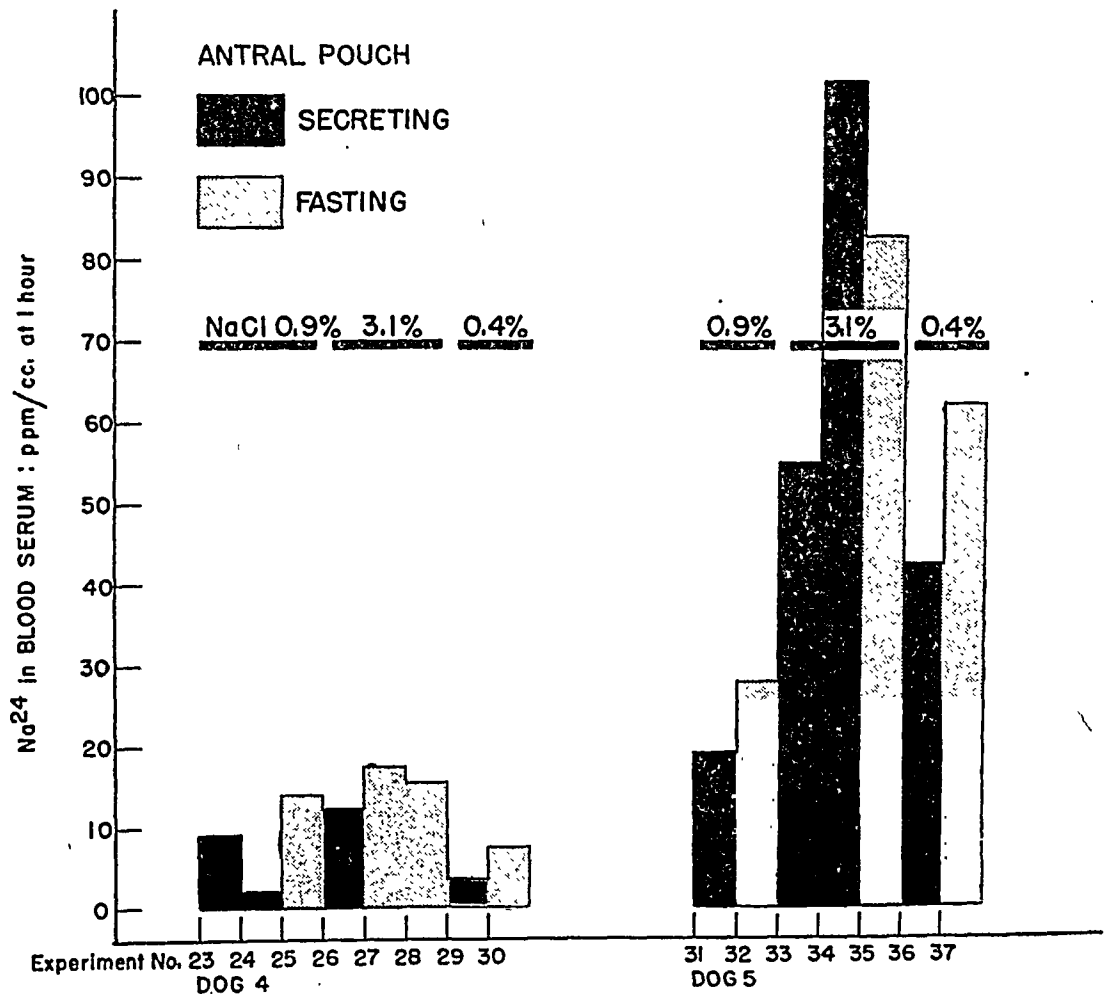


FIG. 2. THE CONCENTRATION OF  $\text{Na}^{24}$  IN THE BLOOD SERUM ONE HOUR AFTER ITS INTRODUCTION INTO THE POUCHES IN THE 2 DOGS WITH POUCHES OF THE ANTRUM OF THE STOMACH

In the experiments represented by the black blocks, the dog's stomach was in an active state of digestion; in those represented by the gray blocks, the dogs were fasting. The percentages of the  $\text{Na}^{24}\text{Cl}$  solution used in the various experiments is given at the upper half of the chart. (The scale of the sodium concentration is ten times that used in Figure 1.)

electrolyte concentrations produced and the concentration of radioactive sodium in the serum.

### 3. Body pouch—Secreting state—Hypo- and hypertonic $\text{Na}^{24}\text{Cl}$ solution

Four experiments using 0.4 per cent  $\text{Na}^{24}\text{Cl}$  solution were made on dogs 1 and 3; and 4 using 3.1 per cent were made on dogs 1 and 2. The dogs were in the secreting state in all experiments. The data are recorded in Table I, experiment numbers 9 through 13, and 20 through 22. The concentration of radioactive sodium found in the blood serum, one hour after the introduction of the sodium, is shown in Figure 1.

There was no significant difference in the concentration of radioactive sodium found in the serum, in spite of the wide range in osmotic tension. The concentrations were comparable to those found in the experiments in group 1, with isotonic saline in the secreting state.

### 4. Antral pouch

Fifteen experiments were made on 2 dogs with an antral pouch, dogs 4 and 5. The data are given in Table II; the concentration of radioactive sodium found in the blood serum, one hour after its introduction into the pouch, is shown in Figure 2 (the scale is 10 times that of Figure 1).

In 5 experiments, the radioactive sodium was in isotonic chloride solution; in 2 of these, the dogs were fasting (experiment numbers 25 and 32), and in the other 3, secreting (experiment numbers 23, 24, and 31). In 6 experiments, 3.1 per cent sodium chloride solution was used, with half in the secreting state (experiment numbers 26, 33, and 34), and half in the fasting (experiment numbers 27, 28 and 35). Hypotonic saline, 0.4 per cent, was put in the pouch for the remaining 4 experiments (numbers 29, 30, 36, and 37), each dog being tested in the secreting and fasting states.

In these experiments, approximately 10 times the concentration of radioactive sodium was found in the serum compared with that in dogs with a pouch of the body of the stomach. In all except one experiment (number 34), the concentration in the serum was greater when the dogs were fasting. The differences between the fasting and secreting experiments are, however, not as great as those encountered with the body pouches.

There is an apparent effect of the osmotic pressure of the sodium solution in these experiments, in contrast to those with the body pouches; the absorption from the hypertonic solution is slightly greater. The differences however are not significant.

If the difference in the surface area of the mucous membrane of the two types of pouch is considered, the relative absorption of sodium from the antrum is increased. The surface area of the antral pouch is approximately one-tenth that of the body pouch. The absorption of sodium per unit of surface area is therefore 100 times greater from the antral than from the body pouch.

## DISCUSSION

The absolute amounts of radioactive sodium absorbed from the stomach are small but not insignificant. From 1 to 7 per cent of the sodium introduced into a pouch of the body area is absorbed in one hour. From 1 to 50 per cent of the sodium introduced into the antral pouch is absorbed during the same period. Sodium is secreted with the gastric juice. It is obvious that, in any study of gastric secretion, the reabsorption of base must be evaluated. The fluid within a pouch of the stomach is the resultant of secretion and reabsorption, and represents contents, not secretion.

It is in keeping with absorption elsewhere in the gastro-intestinal tract that more sodium should be absorbed from the antrum of the stomach than from the body. The absorbing surfaces of the intestinal tract are bathed by a neutral or alkaline fluid. The contents of an antral pouch are neutral or slightly alkaline. In any future consideration of absorption from the stomach, it will be necessary to differentiate between the neutral-alkaline antrum and the acid body. A whole stomach pouch is hardly a suitable medium for the study of secretion or absorption.

The removal of basic ions from the gastric lumen, and their transport across the parietal cell into the body fluids, might be an integral part of the mechanism by which the hydrogen ion is secreted in the reverse direction. If the sodium ion were so transported, more sodium should have been absorbed during the secreting state. These experiments, therefore, tend to exclude such a mechanism for the formation of hydrochloric acid.

It is unknown which cell secretes or reabsorbs sodium. It is inconceivable that the parietal or acid secreting cells could allow a free passage to sodium during the active secretion of acid. A possible explanation of our observations is that the parietal cells become pervious to sodium when they are resting. On the other hand, it is not necessary for the parietal cells to play any role in the reabsorption of the sodium. All of this ion could pass through the pepsin or mucin secreting cells. A smaller amount might well be absorbed during the active secretion of pepsin, which occurs during the secretory phase of the stomach. Such a theory does not hold for the mucin cells, since

the secretion of mucin does not run parallel to that of acid and pepsin.

The lack of effect of varying the osmotic pressure on the sodium absorption suggests that the absorption mechanism is not one of a simple Donnan equilibrium.

#### SUMMARY AND CONCLUSIONS

The absorption of radioactive sodium has been observed in dogs from a pouch of either the body or antrum of the stomach.

Radioactive sodium is absorbed in small but significant quantities from the body or acid secreting area. Two to three times as much is absorbed when the mucosa is in the resting rather than in the secreting state. The gastric antrum absorbs 100 times as much per unit of surface area as the acid secreting body. Whether the stomach is fasting or secreting makes less difference to the absorption from the antrum than to the absorption from the body.

Variations in the osmotic pressure of the sodium solution and in electrolyte concentrations in the blood serum, within the limits observed, had no significant effect on the rate of absorption of the sodium.

The concept that the fluid in an isolated pouch of the stomach represents secretion is obsolete. It is suggested that the antrum plays a preponderant

role in any observed absorption from the whole stomach.

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# GASTRIC SECRETION. III. THE ABSORPTION OF HEAVY WATER FROM POUCHES OF THE BODY AND ANTRUM OF THE STOMACH OF THE DOG

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It is held as axiomatic that an understanding of the mechanism of the passage of substances from the blood stream to the gastric lumen must include a knowledge of the passage of these same substances in the reverse direction. In the previous paper (1), the absorption of radioactive sodium ( $\text{Na}^{24}$ ) from the stomach was reported. Tangible amounts of  $\text{Na}^{24}$  placed in a stomach pouch were found after 15 minutes in the blood serum. From 1 to 7 per cent of the introduced base was absorbed from the acid bearing area during an hour, the amount depending upon whether the stomach was in a resting or secreting state; as much as 50 per cent was absorbed from the alkaline secreting antrum in the same period. The continuous passage of this basic ion into the body fluids altered significantly the concentrations of base in the gastric contents. Since it is probable that the sodium of neutral sodium chloride secreted into the pouch would be reabsorbed in a manner similar to the sodium introduced from the outside, it was concluded that the contents of an isolated pouch represents not true secretion but the resultant of secretion and absorption.

Water is an integral part of any secretion and the study of its exchange across the gastric membrane would seem essential for a comprehension of the control of electrolyte concentration in gastric juice. It has long been established (2, 3) that the contents of a gastric pouch is isotonic with the blood plasma, and it has been assumed, therefore, that the secretion itself is isotonic. It might be that the actual product secreted by a cell is not isotonic but that equilibrium with the internal fluids is rapidly reached by the exchange of water through a neighboring cell. Examples of the ability of the body to handle fluids other than isotonic are not wanting; the intestinal tract can absorb large quantities of water, and the kidney produces urine far to either side of isotonic.

The absorption of water from the whole stomach or one of its parts has not been demonstrated. Many years ago Pavlov showed that water was a mild stimulant of gastric secretion (4). Thus, if water is inserted into an isolated pouch of the stomach, with the passage of time there is a net gain in volume. It is possible, however, that as the solutions of electrolytes are flowing into the stomach and increasing the volume of the gastric pouch contents, there is a simultaneous absorption of a lesser volume of water.

It was decided to answer this question of the absorption of water from the stomach, using deuterium oxide or so called heavy water ( $\text{D}_2\text{O}$ ). This substance can be distinguished from ordinary water ( $\text{H}_2\text{O}$ ) by physical means, and by its greater density, and offers, therefore, the ideal for such an experiment.

Heavy water has been used in other parts of the animal body as a tracer substance to demonstrate the direction and rapidity of shift of water from one compartment to another. Recently Kinsey, Grant, and Cogan (5) have measured by means of  $\text{D}_2\text{O}$  the flow of water into and out of the eye. The rapidity and volume of exchange found was surprising. In rabbits, one half of the water of the aqueous humor was replaced every 2.7 minutes. In the monkey, the exchange was slower, taking 7 minutes for one half to shift. The exchange of water out of the vitreous humor of the rabbit was somewhat slower than from the aqueous.

Animals have been observed to assimilate  $\text{D}_2\text{O}$  much as they do  $\text{H}_2\text{O}$ . Only in high concentrations and over long periods have organisms shown intolerance to  $\text{D}_2\text{O}$  (6). For experiments of short duration or when small quantities are used,  $\text{D}_2\text{O}$  can be considered to be handled by the organism much as is  $\text{H}_2\text{O}$ .

## METHODS

Two large male dogs were used, one with a pouch of the body, or acid bearing area of the stomach, and the

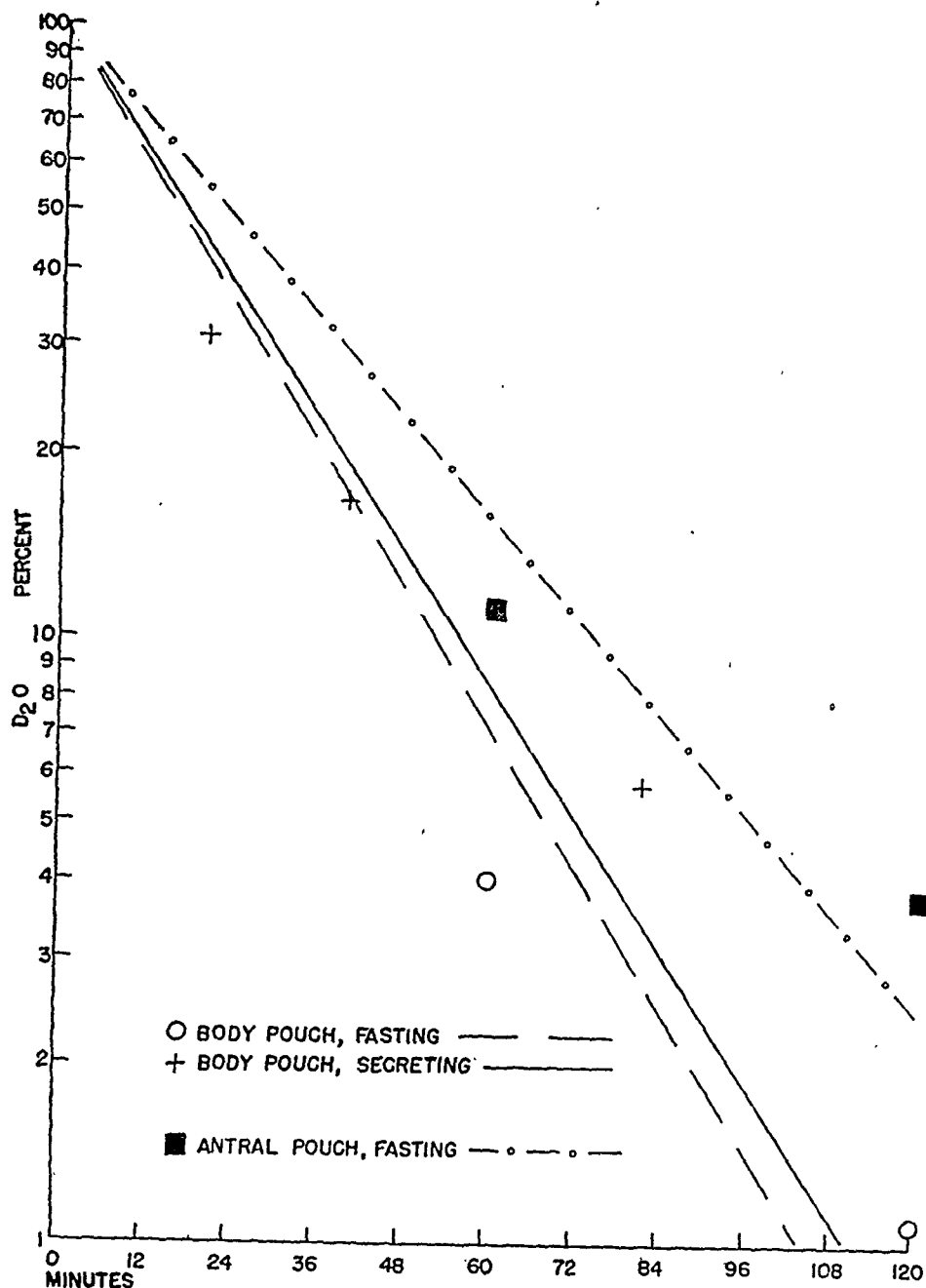


FIG. 1. PERCENTAGE OF  $D_2O$  REMAINING IN BODY AND ANTRAL POUCHES, FASTING AND SECRETING, AT INTERVALS AFTER INSERTION

The percentage is plotted on a logarithmic scale. Fifty per cent of the  $D_2O$  had been absorbed from the pouch at approximately 20 minutes.

other with a pouch of the antrum. The preparation and care of such pouches has been described in previous papers (7, 1).

The exchange of water across the gastric membrane was measured by the rate of absorption of  $D_2O$  from the pouch into the animal. A known volume of  $D_2O$  was inserted into the pouch and its decreasing concentration measured at intervals. The  $D_2O$  determinations were made for us by Dr. Kinsey and Dr. Grant, to whom we are greatly indebted. The details of the method are described in their paper (5). The  $D_2O$  and  $H_2O$  were distilled off from the gastric fluid under partial vacuum. The specific gravity of the distillate was determined by

means of a Linderstrøm-Lang constant density gradient tube.

In the calculation of the percentage of  $D_2O$  which had disappeared from the pouch, that is, had been absorbed into the animal, due account was taken of the volume of the aliquots removed from the pouch for the determinations, as well as the amount of secretion which was flowing into the pouch as measured by the increase in volume of pouch contents.

The  $D_2O$  was made up as 10 per cent of the water of an isotonic  $NaCl$  solution. An isotonic solution was used in order to avoid the secretion of gastric juice caused by water or a hypertonic solution (8). A known quantity

of phenolsulphonephthalein (P.S.P.) was added to the D<sub>2</sub>O salt solution, and its recovery from the pouch at the end of the experiment served as a check on the absence of leakage from the pouch. It has been shown by Wilhelmj (9) that P.S.P. is not absorbed from the whole stomach pouch.

The chemical determinations made on blood and gastric juice were by standard laboratory procedures.

## EXPERIMENTS

Two experiments were performed on the dog with the pouch of the body, or acid secreting area of the stomach. In the first, the dog was fasting; the dog had not been fed for 48 hours but had been allowed free access to water. The resting state of the stomach was proven by

TABLE I

*D<sub>2</sub>O: Percentage remaining in pouches at intervals after insertion*

The dog with the body pouch weighed 21.7 kgm.; the dog with the antral pouch weighed 17.3 kgm.

	Minutes						Date
	20	40	60	80	120	180	
<i>Body pouch</i> Fasting Secreting	31	17	4	6	1	0	December 11, 1941 December 22, 1941
<i>Antral pouch</i> Fasting			11	4	0		December 11, 1941

a period of control observation before introducing the D<sub>2</sub>O solution; during the 4 hours before the onset of the experiment, the dog secreted only 5 cc. of an alkaline juice. Thirty cc. of the D<sub>2</sub>O salt solution were inserted by catheter. At the end of one hour, the entire contents of the pouch, 36.5 cc., was removed, an aliquot of 1.9 cc.

taken for analysis and the remainder returned. At 2 hours, the contents measured 45 cc., and a 2.1 cc. aliquot was taken. At 3 hours, the end of the experiment, the contents measured 5.2 cc. In this experiment, there was a 90 per cent recovery of the P.S.P. After the final emptying of the pouch, 35 cc. of blood were removed from the femoral artery.

The percentage remaining at the hourly intervals of the original amount of D<sub>2</sub>O placed in the pouch is given in Table I and shown graphically (broken line) on a logarithmic scale in Figure 1. The absorption of D<sub>2</sub>O was more rapid than anticipated; indeed, none of the D<sub>2</sub>O was detectable in the pouch contents at 3 hours. From the figure, it is apparent that 50 per cent of the D<sub>2</sub>O was absorbed at approximately 17 minutes.

In order to ascertain the condition of the animal, additional observations were made. The gastric secretion was analyzed for pH and chloride content. An acid-base partition including protein, as well as an NPN and hematocrit, was determined on the blood. The figures are given in Table II; all of the blood findings were within normal limits. The original gastric pouch sample showed a fasting secretion but the later samples were consistent with a small amount of acid secretion.

In the second experiment, the dog was actively secreting hydrochloric acid-pepsin juice. Fed by stomach tube in the morning, 2 hours before starting the experiment, the dog was observed to secrete 74 cc. of acid juice during the hour before the experiment. After emptying the pouch, 30 cc. of the D<sub>2</sub>O salt solution were inserted by catheter. At 20 minutes, 40 cc. of contents were removed and a 2 cc. aliquot taken. At 40 minutes, 77 cc. were found and 2 cc. taken. At 80 minutes, the end of the experiment, 134 cc. were recovered. Ninety-seven per cent of the P.S.P. inserted was retrieved at the end. Thirty-five cc. of arterial blood were taken after the final emptying.

TABLE II

*Gastric secretion and arterial blood studies on dogs with body and antral pouches*

Where two figures are given for gastric secretion, the top one represents the determination done on the sample removed immediately prior to insertion of the D<sub>2</sub>O, the one in italics, the determination done on the sample removed at the end of the experiment.

State of pouch	Gastric secretion		Arterial blood										
	pH	Cl	Hemato-crit	NPN	Serum protein	Cl	CO <sub>2</sub>	PO <sub>4</sub>	Lactic acid	Pyruvic acid	Total base	Na	Ca
<i>Body pouch</i> Fasting		<i>m.eq. per L.</i>	<i>per cent cells</i>	<i>mgm.</i>	<i>grams</i>	<i>m.eq. per L.</i>	<i>m.eq. per L.</i>	<i>mgm.</i>	<i>mgm.</i>	<i>mgm.</i>	<i>m.eq. per L.</i>	<i>m.eq. per L.</i>	<i>m.eq. per L.</i>
	8.2 3.2	118 158	50	25	5.9	111	33	3.0	30	2.4	155	143	5.0
Secreting	1.0 1.0	157 161	53	46	7.3	58	33	5.3	22	4.5	154	145	4.9
<i>Antral pouch</i> Fasting	1.8 2.0	151	55	34	6.8	114	27	3.1	28	1.6	153	143	4.9

The percentage of the  $D_2O$  remaining at each withdrawal is given in Table I and in Figure 1 (unbroken line). The apparent rate of absorption of the  $D_2O$  from the actively secreting pouch did not differ from that observed when the animal was fasting. Sixty-nine per cent of the  $D_2O$  had disappeared in 20 minutes. The curve reconstructed from the three points observed indicates again that 50 per cent was absorbed in approximately 17 minutes.

The gastric juice and blood determinations, made to evaluate the state of the animal, are given in Table II. The gastric pouch juice showed an active state of secretion. The blood findings were within the normal range except for a slightly elevated hematocrit, a high  $CO_2$  content, and low chloride concentration, pointing to partial chloride depletion.

One experiment was run on the dog with the antral pouch. The animal had not been fed for 24 hours prior to the experiment. The juice washed out of the pouch, however, was acid, pH 1.8, suggesting a continued active secretion. Seven cc. of the  $D_2O$  solution were used. Volumes were measured and aliquots were removed at hourly intervals. The final corrected volume was 10 cc., and there was essentially a total recovery of P.S.P. Thirty-three cc. of arterial blood were taken.

The figures for the percentage of  $D_2O$  remaining in the pouch are given in Table II and in Figure 1. At 3 hours, all of the measurable  $D_2O$  had been absorbed from the pouch. The slope of the disappearance or absorption rate of the  $D_2O$  was slightly more gradual than that of the body pouch. The difference is not considered significant, however, in view of the few observations. The reconstructed curve intimates that half of the  $D_2O$  had been absorbed in 23 minutes.

The antral juice and blood findings are recorded in Table II. The juice showed a continued active secretion; all of the blood findings are those of a normal dog.

#### COMMENT

If it is assumed that  $D_2O$  and  $H_2O$  cross the gastric membrane with equal facility and rapidity, then under the conditions of these experiments, half of the water within the pouch crosses the membrane in approximately each 20 minutes. The data available regarding exchange of water elsewhere in the body of experimental animals make such a rapid exchange reasonable. The observation by Kinsey *et al.* (5) that there is a different rate of transfer in the eye of the rabbit compared with that of the monkey suggests that the anatomically comparable cell membranes of certain animals may be more slowly permeable to water than those of others. The slower rate observed for the dog's stomach, as compared with the rab-

bit's or monkey's eye, may be due to the fact that we are dealing with a different membrane, secretory rather than serous, or that it is a different animal.

Although the rate of exchange of water observed crossing the membrane of a gastric pouch in the dog may not be directly applicable to the human or other animal stomach, the observations of this paper certainly point to a rapid physiologic exchange of water in the stomach. The ability of the stomach to reach isotonicity between its contents and the body fluids is explained, and it lends a broader interpretation to the observations that water (4) and hypertonic fluids (8) are stimulants of gastric secretion.

#### SUMMARY AND CONCLUSIONS

The exchange of water across the gastric membrane has been investigated in the dog by observing the absorption of heavy water, deuterium oxide ( $D_2O$ ), from stomach pouches. One animal had a pouch of the body, or acid secreting portion of the stomach, and another a pouch of the gastric antrum. Under the conditions of the experiments, half of the  $D_2O$  was absorbed in approximately 20 minutes.

There was no significant difference in the rate of absorption between the body and antral pouches. Whether the body pouch was in a secreting or resting state also made no difference.

If it is assumed that the gastric membrane handles  $D_2O$  as it does  $H_2O$ , it can be concluded that there is a rapid exchange of  $H_2O$  between the gastric contents and body fluids; such an exchange would be a major reason for their isotonicity.

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# A COMMUNITY STUDY OF CARRIERS IN EPIDEMIC POLIOMYELITIS<sup>1</sup>

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Since 1937, simple methods for the detection of poliomyelitis virus in human stools (1 to 4) have proved useful in case finding. The effectiveness of these methods has broadened our ideas about the way the virus is distributed in familial (5, 6), institutional (7), and community (8, 9) outbreaks of poliomyelitis. The evidence adduced has indicated that the virus can be found in human stools under specified conditions, *viz.*, in paralytic, abortive, and ambulatory convalescent cases of poliomyelitis, and in presumably healthy contacts, during epidemic periods.

The next step should be to determine the distribution of the virus in the whole population at risk during natural epidemics of the disease. This has not as yet been reported. Opportunity for such a "herd study" occurred in the State of Alabama during the fall of 1941.<sup>3</sup> The present report concerns the examination of 176 of 181 persons, living in a defined community, affected in the extension of a widespread epidemic of poliomyelitis, to determine the carrier rate in a post-epidemic period.

## MATERIAL AND METHODS

**Clinical material.** In the summer of 1941, a widespread epidemic of poliomyelitis occurred in the state of Alabama. In mid-August, one of us (A. E. C.), already at work in the field, called attention to the presence of a heavy concentration of cases in the small town of Cordova (pop. 1670), Walker County, Alabama.<sup>4</sup> Shortly thereafter, a geographically defined community on the eastern cor-

<sup>1</sup> Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

<sup>2</sup> Frank Lusk Babbott, Jr. Fellow in Pediatrics.

<sup>3</sup> For the privilege of making these studies, we are indebted to the late Dr. J. N. Baker, former State Health Officer, Montgomery, Alabama; and especially to Dr. A. M. Waldrup, Walker County Health Officer, Jasper, Alabama. The assistance of these health officers and their staffs is gratefully acknowledged.

<sup>4</sup> In the summer and fall of 1941, 871 reported cases of poliomyelitis occurred in the State of Alabama. Of these 871, 125 cases with a 2 per cent mortality occurred in Walker County (pop. 67,000) Alabama.

ridor of Cordova, containing most of the town's cases, was selected for study. Facilities for a combined clinical and laboratory study were provided, and after September 6, 1941, clinical histories of all, and stool specimens from 176 persons, were obtained.

**Character of the community.** The community selected for study, though situated on the outskirts of Cordova can be described as semi-rural, but over-crowded houses and outbuildings containing various domestic animals were close together. The houses were largely poor wooden structures. Surface privies of poor construction were almost universally present.

The inhabitants were mainly coal miners, and cotton-mill workers. A diagrammatic schema of the community studied is shown in Figure 1.

**Course of epidemics.** Six cases of poliomyelitis from this community were reported to the Walker County Health Department during July and August 1941. Their distribution appears in Figure 2.

**Collection of stools.** Between August 15 and August 25, 1941, one of us (A. E. C.) collected stools in 50 per cent glycerol from 6 children in the community, all suspected of having had poliomyelitis. Between September 6 and September 15, stools were obtained from 74 persons. Stools from 163 persons were collected before September 20, and of the remaining 18, 13 provided stools before October 6. Stools from 5 adults were not obtained. One other stool was collected on November 11.

Stools were obtained under fairly uniform conditions. In a house to house canvass, the essential articles, and careful instructions for collecting each stool specimen, were left with some responsible member. Infants' and young children's stools were collected in individual sterilized pots, and then transferred to a clean cardboard container. All other stools were deposited on wax paper, and then placed in the container. Stools were collected within 12 to 24 hours, stored in a refrigerator at 6° C., and held there usually 1 to 5 days before etherization. A small number of stools remained in the ice-box for 10 to 20 days, and the glycerinated stools remained there for 50 to 80 days before extraction.

**Preparation of inoculum.** Roughly a 15 per cent aqueous stool suspension was divided into two fractions, (a) an untreated suspension for intra-nasal instillation, and (b) an etherized fraction (10 per cent ether by volume added to the 15 per cent stool suspension, which was allowed to stand in contact at ice-box temperature overnight) from which the middle layer was obtained after centrifugation for intraperitoneal inoculation.

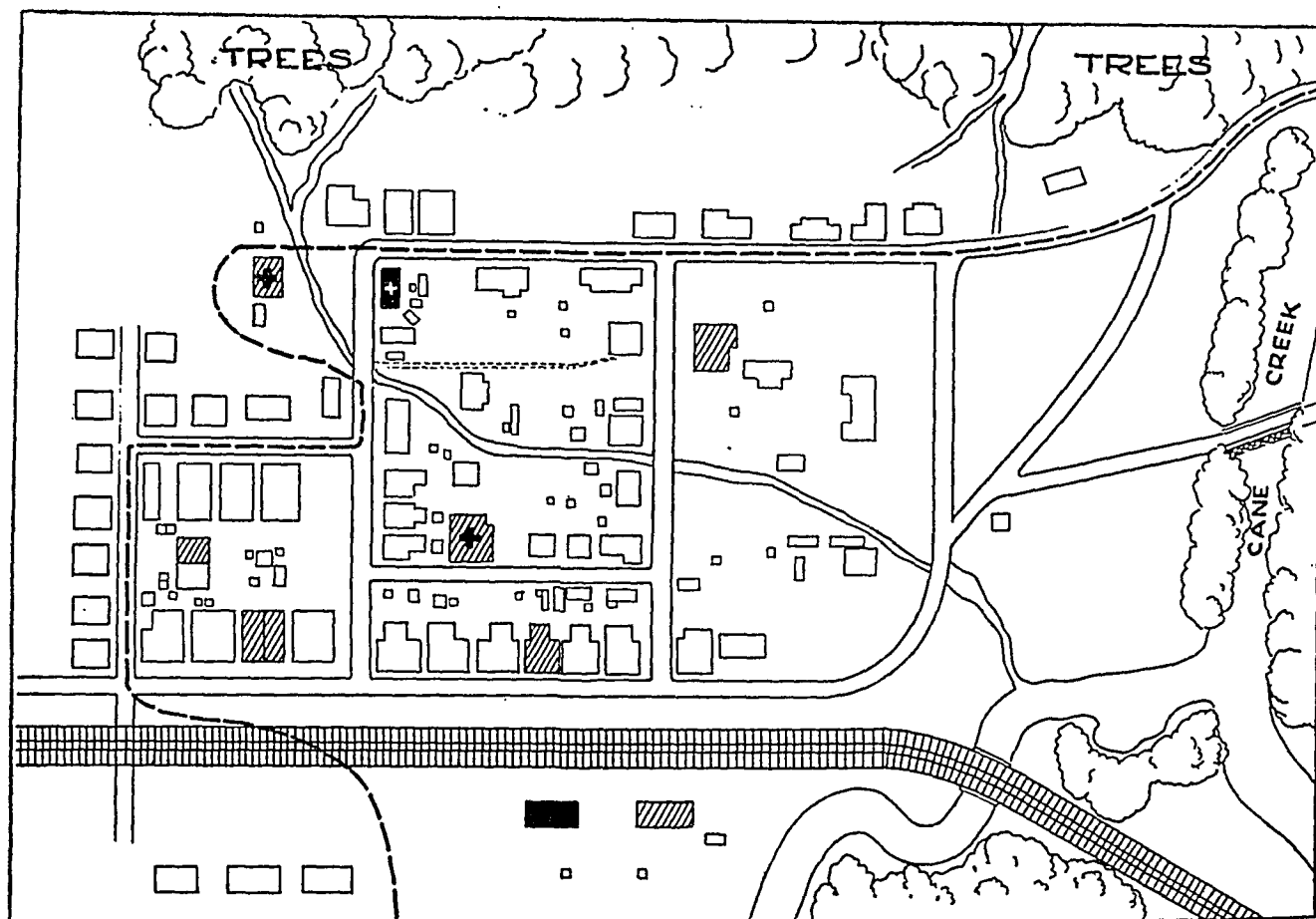


FIG. 1. DIAGRAM OF COMMUNITY STUDIED

The community is outlined by the broken line and the lower and right hand margins of the figure; the area is roughly  $1000 \times 600$  feet. The black blocks represent houses in which paralytic poliomyelitis occurred; the shaded blocks represent houses in which abortive poliomyelitis occurred. The residence of individuals providing positive stools is indicated by a cross.

Two types of inocula were used, (1) pooled stool inocula, i.e., 1.5 cc. of fraction (a), and 5.0 cc. of the etherized fraction (b), each from 4 individuals in the same age group. These were pooled for use in one monkey (47 pools in all). (2) Individual stool inocula, viz., 6.0 cc. of fraction (a), and 20.0 cc. of fraction (b) were composed of stools from one person. These were

inoculated into at least one monkey (25 stools in all). Glycerinated stools were extracted in the same manner as raw stools.

**Inoculation.** The combined intraperitoneal and intranasal portals were used for inoculation. Each monkey received 10.0 to 20.0 cc. of the etherized fraction (b) once intraperitoneally; and also 2.0 cc. of raw stool extract, fraction (a), were dropped into the nasopharynx of each animal on each of 3 consecutive days.

**Animals.** *Macaca mulatta* weighing roughly 1.5 to 3.5 kgm. were used. Inoculated animals were exercised daily, and daily rectal temperatures charted for 5 weeks unless the animal was killed earlier. Ten monkeys were re-inoculated after 5 weeks of uneventful clinical observation.<sup>5</sup> All animals which showed symptoms of poliomyelitis, or remotely suggestive of poliomyelitis, were sacrificed at what appeared to be an appropriate time. All other monkeys were sacrificed at the end of the 5 week experimental period. Olfactory bulbs, midbrain, medulla, and cervical, thoracic, and lumbar regions of the spinal

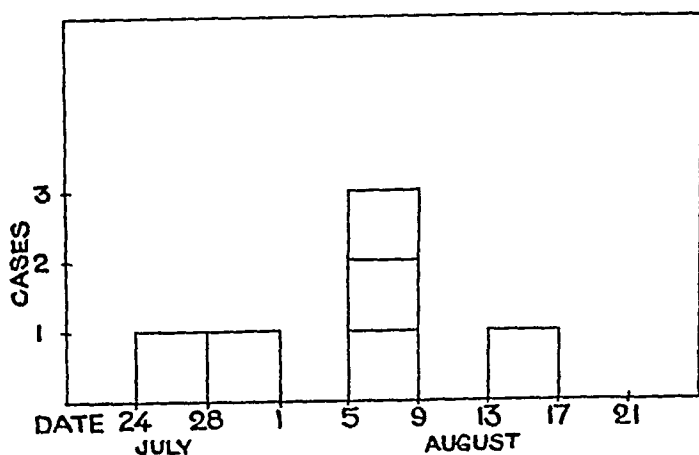


FIG. 2. REPORTED CASES OF POLIOMYELITIS WHICH OCCURRED IN THE COMMUNITY

<sup>5</sup> Monkeys used for second tests were tuberculin tested using 10.0 mgm. of human old tuberculin in the test dose. Only tuberculin negative animals were used.

cord were preserved in 10 per cent formalin, and in 50 per cent glycerol.

**Criteria for identification of poliomyelitis virus.** The criteria adopted for the identification of the virus were: (1) the production of experimental poliomyelitis in monkeys, viz., after a variable incubation period, usually 5 to 15 days, the development of fever, excitement, tremor, weakness, and paralysis, the latter being generally associated with a fall in temperature; and (2) histological lesions typical of poliomyelitis in the lumbar and cervical cords.

Passage of the virus to a second monkey was accomplished with one strain, but was not considered to be an essential criterion under the conditions of the present study.

## RESULTS

**Accidental losses.** Two monkeys died prematurely, one of purulent peritonitis, the other of an abscess of the abdominal wall. These tests were

completed in other monkeys with negative results. One monkey died at the end of the experimental period, in the 5th week, of pulmonary tuberculosis.

**Completed tests.** One hundred and ninety stools from 176 individuals were subjected to 81 tests, of which 8 were positive. Three of these were from 47 pools; 4 of the 8 were from the subsequent fractionation of the pools, and the remaining positive was also from a single stool specimen. Altogether there were 3 individuals with positive tests. The results are amplified below. The first child represents an abortive attack of poliomyelitis.

**FAMILY H (Figure 3).** Paul H., male, *aet.* 2 years, one of 4 siblings. Birth history was uneventful. Whooping cough at 6 months was followed by pneumonia. He had recurrent pneumonia at 1 year, with prolonged convalescence during which time he had "colitis" with bloody diarrhea. On June 30, through July 4, 1941, his mother, *aet.* 25 years, had fever, headache, and sore throat. From July 10 to July 20, a brother, *aet.* 6 years, had suppurative inguinal lymphadenitis. On August 4, Paul H. was irritable, and refused to eat. The next day he was feverish, found fever, stiff neck, red throat, and cervical adenitis. On August 4, his neck was still stiff, and he vomited once. He was confined to bed for 6 days, but thereafter seemed well. A stool specimen from Paul H. obtained August 26, and inoculated 77 days later into monkey A-69, was positive. A-69 developed fever on the 14th day, paralysis on the 18th day, and was sacrificed on the 20th day after inoculation. Typical histological lesions were found in the cord. Stools collected on September 18, and on October 6, provided negative results.

The second child represents a paralytic case.

**FAMILY N (Figure 4).** Manley N., male, *aet.* 3 years, one of 8 siblings. Birth history was uneventful. He had whooping cough at 1 year. During 1940 and 1941, he had frequent upper respiratory infections with visible swelling of lymph nodes in the neck. A sister, *aet.* 14 years, from July 8 to July 11, 1941, had fever, vomiting, diarrhea, and headache. On August 13, Manley N. had rhinorrhea, and seemed feverish. After a day in bed, he felt well enough to get up and play with his siblings. He was fretful for the next few days, and on August 19, appeared listless and vomited. Two days later a physician found fever of 101° F., a stiff neck, left cervical adenitis, a rapid pulse, and drowsiness. Paralysis of the left leg was observed on August 23. He was examined by one of us (H. A. W.) on December 4. No paralysis was detectable. Stools collected August 26, and September 8, were positive. The first stool preserved in glyc-  
erine, and inoculated 77 days after collection, produced

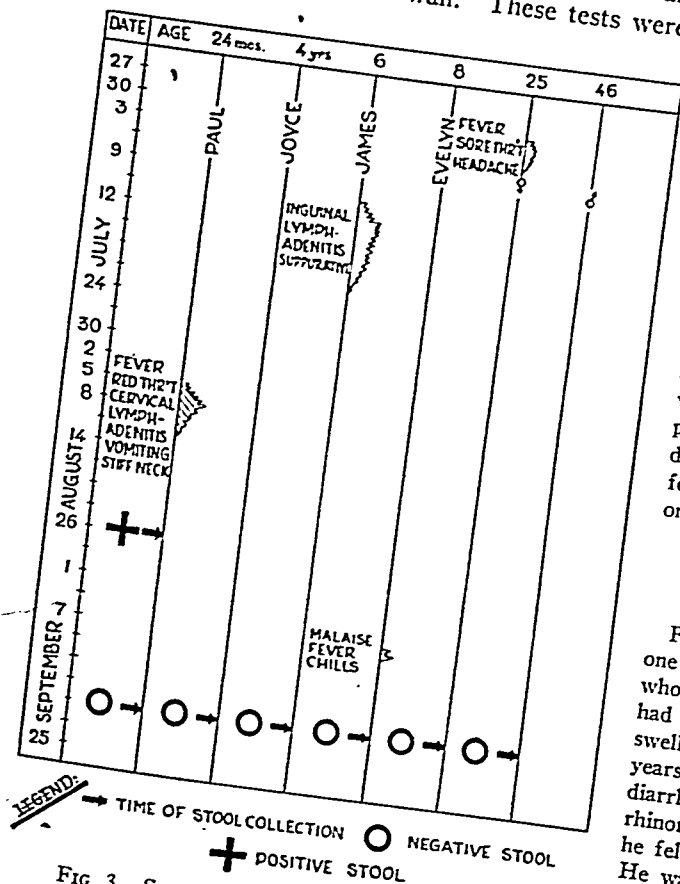


FIG. 3. SCHEMATIC DIAGRAM OF FAMILY H

The vertical lines represent different individuals of the family; their ages appear at the top. The shaded area indicates the time of onset and course of an abortive attack of poliomyelitis; the open area indicates febrile illness.

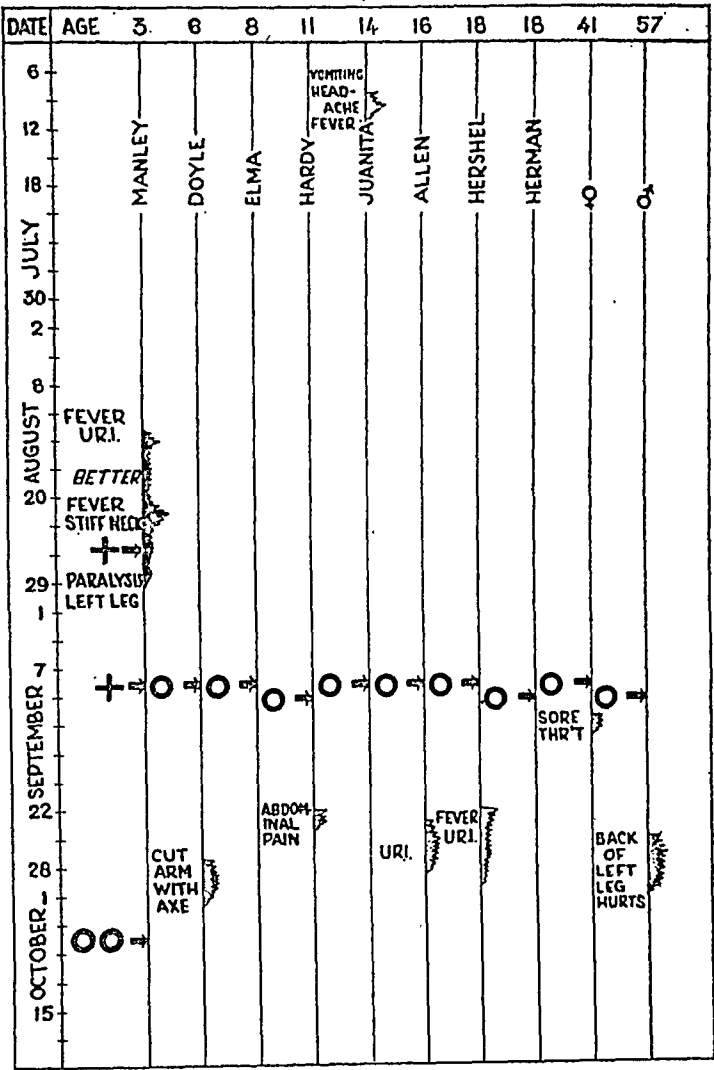


FIG. 4. SCHEMATIC DIAGRAM OF FAMILY N

The legends are the same as in Figure 3. The black area indicates the time of onset and course of a paralytic attack of poliomyelitis; the stippled areas indicate poorly defined illnesses.

fever in monkey A-70 on the 11th, and paralysis on the 14th day. The second stool produced fever in monkey A-45 on the 13th, and paralysis on the 15th day. The monkeys were sacrificed on the 18th and 19th days, and lesions typical of poliomyelitis, found in the cords of both. A stool collected October 6, 1941, was negative.

The third child with positive stools represents an ambulatory convalescent carrier, with a poorly defined antecedent illness.

FAMILY V (Figure 5). Lamarr V., male, *act.* 6 years, one of 3 siblings. Birth history was uneventful. He had bronchopneumonia at 2 years, and whooping cough at 3. A sibling, *act.* 9 years, had fever, headache, and stomach-ache for 3 days beginning July 16, 1941. A physician believed him to have the prodromal signs of poliomyelitis. Another sibling, *act.* 3 years, had 2 days of diarrhea in the first week of August. Lamarr V. was apparently

well, except that his mother thought him fretful during the last week or 10 days in August. Stools collected September 8, and October 6 were positive. The first stool produced fever in monkey A-47 on the 10th day, and paralysis on the 11th day. The second stool produced fever in A-22 on the 14th day, and afterwards, marked ataxia and the development of weakness of the left leg on the 18th day were observed. The monkeys were sacrificed on the 14th and 19th days, and histological lesions typical of poliomyelitis found in both animals. A third stool obtained November 14, 1941, was negative.

To summarize, then, of 190 stools tested, 5 from 3 individuals (2 to 6 years of age) contained enough virus to produce experimental poliomyelitis in 8 rhesus monkeys. Subsequent stools from these individuals were tested until negative. The period of carriage of the virus in the stools was at least 42 days (Lamarr V.) and 26 days (Manley N.). None of the adults' stools were positive.

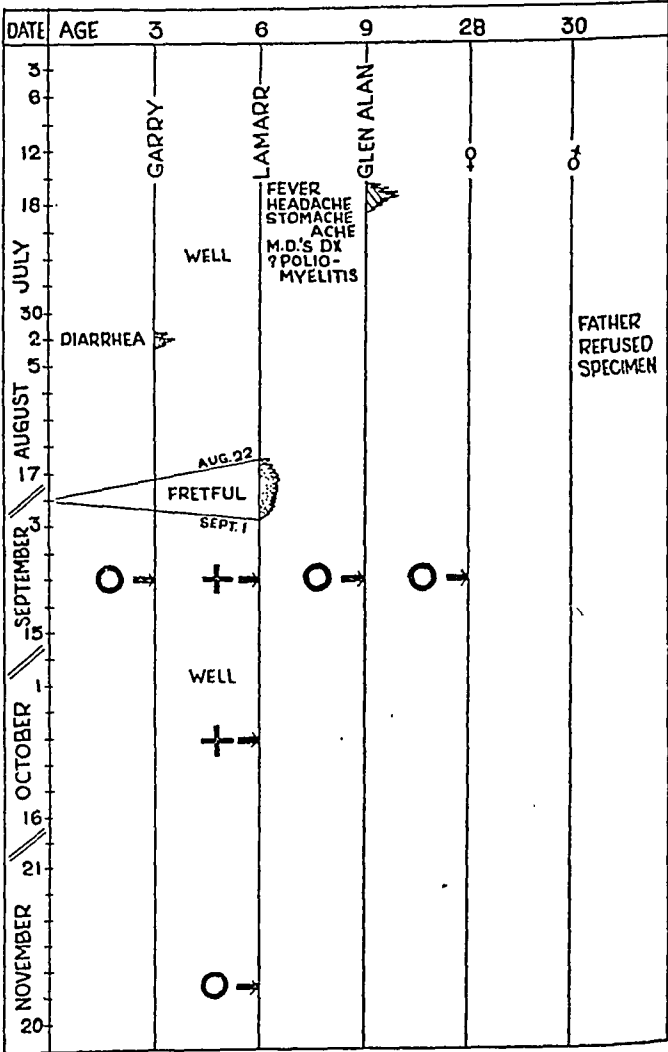


FIG. 5. SCHEMATIC DIAGRAM OF FAMILY V

The legends are the same as in Figures 3 and 4.

## CLINICAL RESULTS

*Classification of cases.* Illnesses which occurred in the community during the summer were classified on the basis of the following terminology: (1) paralytic *viz.*, cases with paralysis developing in association with the usual signs of poliomyelitis; (2) abortive, *viz.*, cases affording a definite history of meningitic or myelitic signs (frequently affirmed by the attending physician), as well as fever, headache, vomiting, and other associated signs and symptoms; (3) suspected abortive, *viz.*, cases with histories of febrile illnesses, usually associated with sore throat, vomiting, diarrhea, and headache, appearing in epidemic times, and not attributable to any other disease; (4) poorly defined illnesses, characterized in the main by the same symptoms as in the suspected abortive, with the exception that there was no known fever, and the illness was of brief duration, usually but for a day; and (5) other diseases,

*viz.*, the exanthemata, tuberculosis, thrombophlebitis, and degenerative diseases.

*In respect to time.* The seasonal distribution of all of these illnesses (in both the adult and juvenile population) is shown in Figure 6. Poliomyelitis present in surrounding neighborhoods was first suspected in the community when one of the juvenile members became ill July 16, 1941. Thereafter, a wave of illness spread rapidly through the population, affecting the juvenile members worst. It reached the peak of its group incidence between August 1 and August 10, 1941.

*In respect to age.* Figure 7 delineates the clinical findings in the community from June 15 to September 15, 1941, compared to the population at risk, at various ages.

*Adult population.* A history of illness corresponding to one of the first four types in our classification was obtained from 33 of 117 adults. Two persons afforded histories only remotely suggestive of abortive poliomyelitis; 8 had febrile

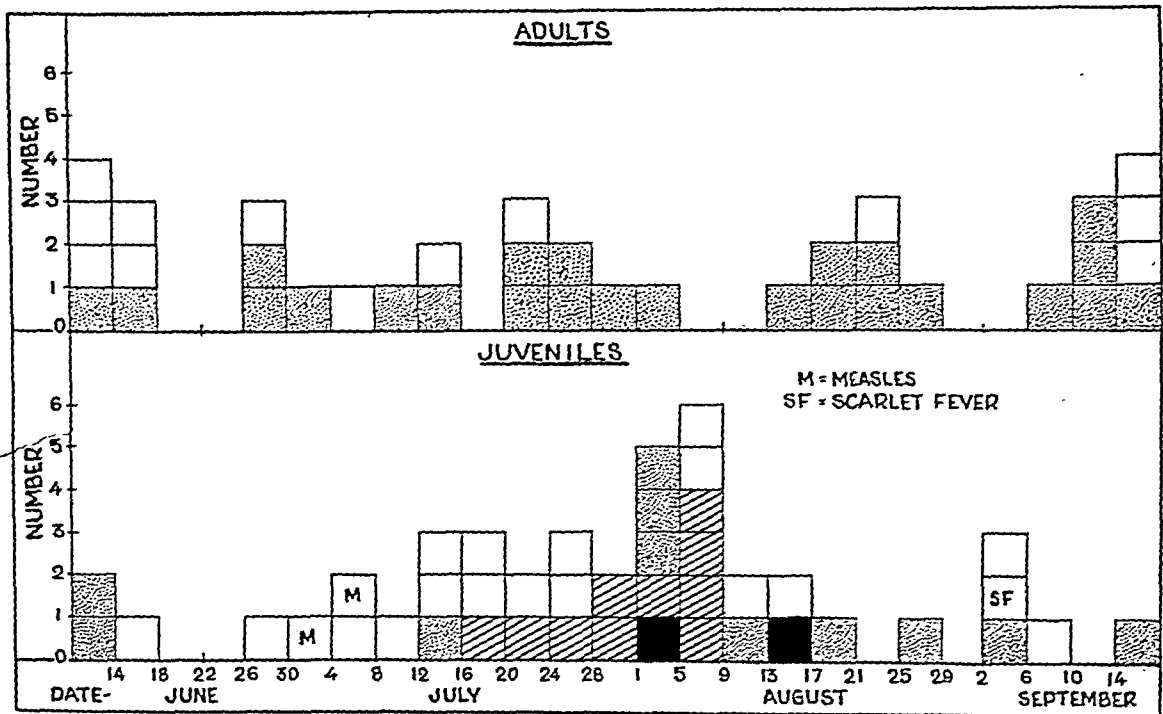


FIG. 6. THE SEASONAL DISTRIBUTION OF ALL TYPES OF ILLNESS IN THE ADULT AND JUVENILE POPULATIONS DURING THE SUMMER OF 1941

Black squares represent paralytic cases of poliomyelitis; shaded squares, abortive cases of poliomyelitis; open squares, febrile illnesses; and stippled squares, poorly defined ailments.

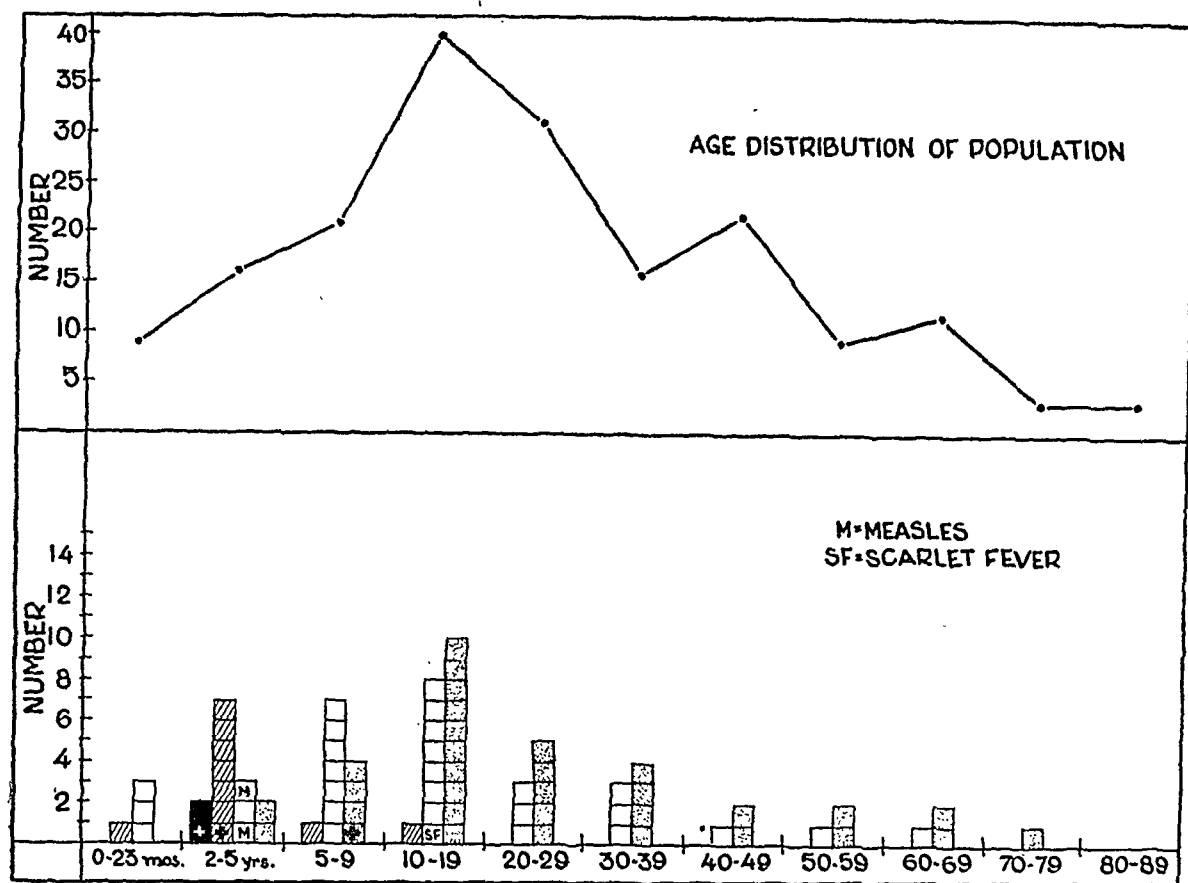


FIG. 7. AGE DISTRIBUTION OF THE POPULATION AT RISK (TOP PANEL) COMPARED WITH THE AGE DISTRIBUTION OF THE COMMUNITY ILLNESS (LOWER PANEL) DURING THE PERIOD OF STUDY

The small crosses indicate cases in which positive stool specimens were obtained. Otherwise the legends defining the blocked areas are the same as in Figure 6.

illnesses, usually with sore throat; and 23 had poorly defined ailments, characterized by rhinorrhea, abdominal pain, or headache.<sup>6</sup>

**Juvenile population.** In the juvenile group, 41 of 64 members had histories of one or more illnesses during the time from June 15 to September 15, 1941. Two children (2 and 3 years of age) had paralytic poliomyelitis; 10 individuals (1 to 12 years of age; average age, 4 years) had abortive poliomyelitis; and 17 persons (1 to 14 years of age; average age, 8 years) had febrile illnesses. Two children had measles, and one other scarlet fever. The remaining 9 of the 41 had ill-defined complaints characterized by headache, vomiting, diarrhea, or sore throat.

<sup>6</sup> Actually there were more adult illnesses than are indicated. One person had pulmonary tuberculosis; another acute thrombophlebitis; one other carcinoma, of which she died in November. Three persons received surgical care of major nature during the summer.

#### ANALYSIS OF RESULTS

*In respect to method.* When so few of our monkeys showed signs and symptoms of experimental poliomyelitis, it was suspected that pooling of stools was disadvantageous. To determine if this were so, stools from 10 of the 12 individuals in the community who provided histories of paralytic or abortive poliomyelitis were each given to at least 2 monkeys. The stools from these 10 persons had been negative previously in their respective pools. In the testing of these stools, 23 animals were used. All of these tests proved negative.

*In respect to clinical material.* Reference to Figure 7 shows that all of the paralytic, and 6 of the 10 abortive cases, occurred in the 2 to 5 year age group. Two of the 3 children with positive stools were in this age group; the third positive was 6 years of age. The greatest incidence of febrile illnesses occurred in the 10 to 19, and 5 to 9

year age groups, in that order. The large number of poorly defined illnesses occurring in the 10 to 19 year old age group is striking, although its meaning is not entirely clear.

The observed ratio of abortive to paralytic cases was 5 to 1. The true ratio, considering some but not all of the 17 febrile illnesses as abortive cases, was probably greater than the observed ratio would indicate.

Critical examination of the illnesses in the adult group (Figure 6) shows in general that they do not have any real relationship, either as to type or to the seasonal distribution, when compared with the illnesses in the juvenile population. None of the adults had a positive stool test, notwithstanding the testing of 112 stools, almost twice the number tested in juvenile group.

*In respect to time of illness and stool collection.* The temporal relationship between the onset of illnesses, stool collections, and the results of stool tests in all members of the juvenile group is out-

lined graphically in Figure 8. Attention has been called to the narrow time zone from July 16 to August 13, 1941, during which the onset of all recognized poliomyelitis cases occurred. At the time of stool collection, the 2 individuals with paralytic poliomyelitis were convalescent in the 4th and 5th week from onset, and the 10 abortive cases were in the 5th to 7th week.

Excepting the glycerinated stool specimens,<sup>7</sup> 82 days, at most, elapsed between the onset of the first illness, July 16, and the collection of the last stool specimen on October 6. At least 24 days intervened from the onset of the last poliomyelitis case, August 13, and the beginning of stool collection, September 6. If one would consider only

<sup>7</sup> It is of interest that 2 of the 6 glycerinated stool specimens which were kept in the refrigerator for 50 to 80 days were positive. Another glycerinated stool from an unidentified member of the community, which was kept in the refrigerator for 90 days produced experimental poliomyelitis in one of the monkeys.

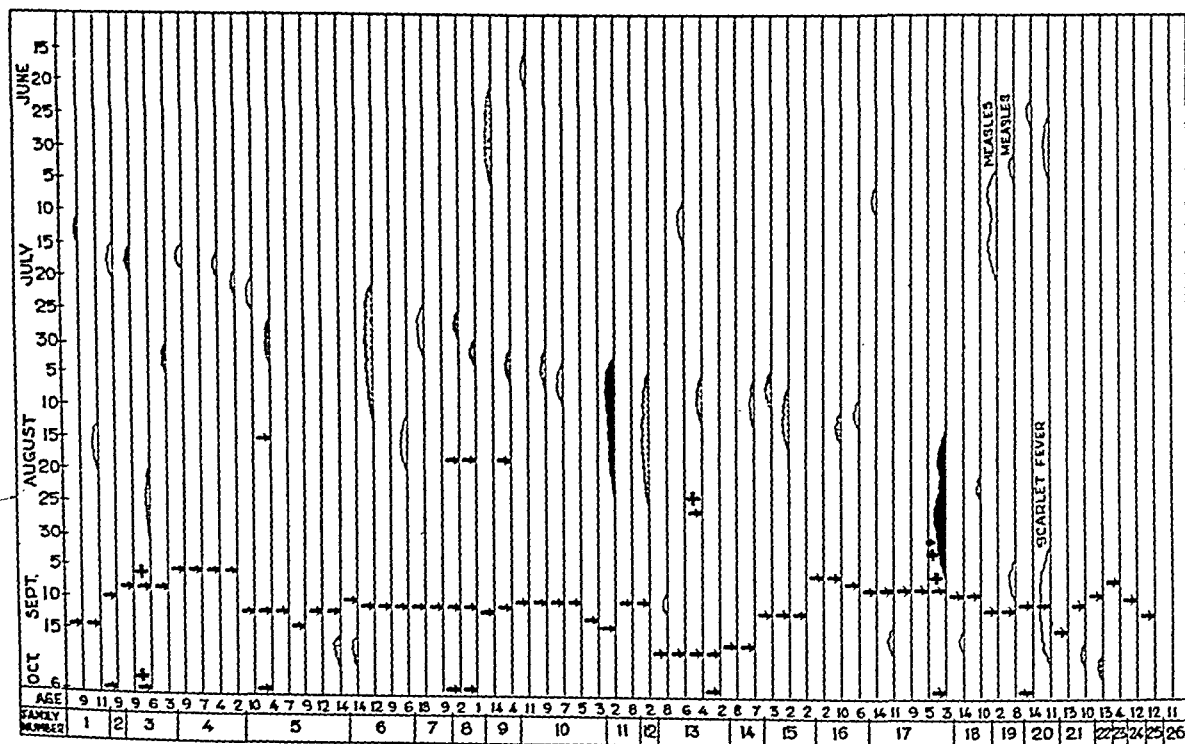


FIG. 8. DIAGRAM OF THE JUVENILE MEMBERS OF THE COMMUNITY

All of the community children under 15 years of age appear as vertical lines. The onset and duration of illness in children ill during the summer is indicated by elevated areas appearing on the respective vertical lines. The type of illness in each individual is indicated by the same markings used in previous diagrams. The black arrows indicate the time of stool collection; the plus signs indicate positive tests.



the children who had clinical poliomyelitis, we find that an average of 42 days elapsed from the time of onset of illness in each, and collection of their respective stools. At the most, it was 52 days and at the least, 26 days that passed.

*In respect to time of illness and positive stools.* Of the children with positive stools, 2 (Manley N. and Lamarr V.) were among the last of those ill in the community during the epidemic period, *viz.*, August 13 and August 25, respectively. The virus was detected in Manley N. on the 13th and 26th days after the onset of his illness, but not on the 54th day. Lamarr V.'s stools were positive on the 14th (if one arbitrarily dates the onset of his poorly defined illness on August 25th) and on the 32nd, but not on the 78th day. The third person yielding the virus (Paul H.), ill at the height of the epidemic, *viz.*, August 5, was positive on the 21st, but not on the 44th day from the beginning of his illness. Had we not had his stool in glycerol, collected August 26, and had we depended only on the September 18 stool, we would have missed detecting the virus in this individual.

*Correlation of clinical and laboratory data.* A rough correlation, considering the limitations of the present methods, can be drawn between the presence or absence of virus in stools, and the span of time elapsing between the onset of illness and stool collection in the members of the juvenile population. It would appear that there is a progressive decline in the number of carriers in the population corresponding with the decline of the disease.

Probably we began our tests too late to detect the maximum number of carriers. Our tests were begun 52 days after the onset of the epidemic, and 24 days after it had waned. The stool collections were between 82 and 24 days after the onset of the first and last recognized cases. In the majority of reports from the literature, the usual carrier period has been 3 weeks.

#### DISCUSSION

Past clinical epidemiological studies (1, 10, 11) have emphasized a widespread distribution of poliomyelitis virus in the population at risk. It has been suggested that abortive cases of poliomyelitis outnumber at any time the paralytic cases, and that they, as well as healthy intermediary con-

tacts, abet the spread of poliomyelitis by contact with susceptible individuals. Evidence obtained by use of the stool method (5, 6) has supported the clinical supposition that mild illnesses, occurring during epidemics, are abortive attacks of poliomyelitis. However the demonstration of a widespread distribution of the virus in "healthy contacts" by the use of these methods is quite recent.

In the past year, Piszczek, Shaughnessy, Zichis, and Levinson (8) and McClure and Langmuir (9) have described an unusually large number of healthy carriers. Some of these carriers were adults. This is not surprising in respect to a prevailing clinical suspicion, which it supports, that the virus is widespread among a large number of human hosts in epidemics. However, others (5, 6), in previous studies, have seldom been able to detect the virus in persons without a history of at least a poorly defined illness, and almost invariably children were the carriers of poliomyelitis virus.

In the present report, our results support the observations that the prolonged presence of the virus is usually associated with a current or antecedent illness, especially in children in the 2 to 5 year age group. Our results are partially at variance in two respects from the studies of Piszczek *et al.*, and McClure and Langmuir: (1) None of our adults provided a positive stool test, and (2) all of our carriers had histories of past illnesses, mild in one to be sure, so that to attempt to differentiate between true "healthy carriers," and convalescent carriers is difficult at times.

There were, however, significant differences in method, bearing on the results of these other recent studies, which should be clarified. These are: (a) that the time of stool collection in respect to the onset of the epidemic was early as compared to the late time of stool collection in ours. In McClure and Langmuir's report, stools were collected from 5 to 24 days (average time 13 days) after the onset of the epidemic, compared with 49 and 82 days (average time 66 days) in ours. In the study of Piszczek *et al.*, "stools were collected within the month after the onset of the disease" (8) in practically all the individuals examined. (b) Our study concerns the distribution of carriers in the whole population at risk. The other recent studies are concerned chiefly with the distri-

bution of the virus in specified hosts. (c) The technical methods used differed in several details.

Further studies directed toward the detection of the virus of poliomyelitis are necessary to determine the natural distribution of poliomyelitis virus, and if, in the future, one were to detect the largest number of carriers possible, several things should be accomplished. These are: (a) the collection of stools before the epidemic has waned, (b) the use of a more susceptible test animal, *i.e.*, *M. cynomolgus*, and (c) the use, if possible (and if they are developed), of more delicate technical methods. Probably the methods used in our study are inadequate, in so far as delicacy is concerned, for the detection of *all* carriers, but under the conditions of the present study, in a post-epidemic period, it seems unlikely that many carriers were missed from the population at risk. We may logically conclude therefore that the virus does not remain "active" in the stools of most persons for more than a few weeks.

#### SUMMARY

1. A community has been studied to determine the distribution of poliomyelitis in the population at risk in a post-epidemic period. Stools from 176 of 181 persons were tested to determine the carrier rate for poliomyelitis virus in the adult and juvenile population.

2. Stools containing the virus of poliomyelitis were detected in 3 children, from 2 to 6 years of age. None of the adults' stools were positive.

3. Though evidence of poliomyelitis was widespread in the human herd, only individuals ill at the end of the epidemic provided positive stools, indicating that the ease of detection of the virus in the herd at risk diminishes with the decline of the epidemic.

For valued advice given during this study, appreciation is gratefully expressed to the late Doctor James D. Trask.

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# THE COAGULATION DEFECT IN HEMOPHILIA: A COMPARISON OF THE PROTEOLYTIC ACTIVITY OF CHLOROFORM PREPARATIONS OF HEMOPHILIC AND NORMAL HUMAN PLASMA<sup>1,2</sup>

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It has been shown that, following the treatment of normal, human, cell-free plasma with chloroform, a proteolytic enzyme is elaborated (1). It has also been shown that the enzyme is associated with the globulin fraction of the plasma proteins (1, 2). Furthermore, it has been demonstrated that the enzyme may be prepared by the action of chloroform on a saline solution of human plasma euglobulin (1). Because hemophilic blood is known to have a deficiency in the coagulation activity associated with plasma euglobulin (3, 4), it seemed advisable to compare hemophilic and normal human plasmas as sources of enzyme activity.

The present communication concerns a comparative study of the proteolytic activity of normal and hemophilic plasma after treatment with chloroform.

## EXPERIMENTAL

Fifty ml. of blood were aspirated into a syringe containing 0.5 ml. of 20 per cent potassium oxalate. The plasma was removed by centrifuging at 2,500 r.p.m. It has previously been shown that removal of blood platelets had no effect on the potency of the plasma enzyme, but in some instances, the plasma was recentrifuged at 4,200 r.p.m. in an angle head centrifuge. This latter operation rendered the plasma essentially cell-free. Plasma was prepared from 7 normal individuals and 7 cases of hemophilia. In each experiment, one normal and one hemophilic plasma was handled simultaneously and treated

under exactly duplicate conditions. The patients were proven cases of hemophilia with a venous blood coagulation time, for 2 cc. of blood, greater than one hour which is about 6 times normal.

The plasma was transferred to 50 ml. glass bottles and  $\frac{1}{10}$  the volume of chloroform added. The mixture was vigorously shaken for one minute and allowed to stand at room temperature until the clot which formed had undergone lysis. It should be noted that both the hemophilic and normal plasmas clotted after the addition of the chloroform in about the same time. In both instances, less than 16 hours were required. The time for complete dissolution of the clot was recorded. The data of the 7 experiments are given in Figure 1. It will be observed that in each instance it took much longer for the clot in the hemophilic plasma preparation to disappear than in the normal plasma preparation. However, there was no direct relation between the coagulation time of the hemophilic blood and the time required for dissolution of the clot. On the average, it took approximately twice as long for clot dissolution in the case of hemophilic plasma preparations as in the case of normal plasma preparations. It will be observed that the same results obtain whether the platelets had been removed from the plasma or not.

It has previously been shown (1, 5) that when human plasma is shaken with chloroform, a clot is formed. When this clot has entirely dissolved, the chloroform plasma preparation contains an active proteolytic enzyme. The activity of a given preparation can be readily measured by determining the rate of disappearance of a given quantity of fibrinogen. The disappearance of fibrinogen can be determined by the addition of an active thrombin solution to the fibrinogen solution. Accordingly, to a given quantity of fibrinogen in solution, 0.5 ml. of chloroform plasma preparation was added, and the time required for the complete disappearance of fibrinogen determined by the addition of thrombin. The time from the addition of the chloroform plasma to the time when no further fibrinogen remained was recorded. The temperature was held constant at 37.5° C. In this way, the proteolytic activities of chloroform preparations of normal and hemophilic plasma were compared. The data are presented in Table I. It will be observed that the normal chloroform plasma preparations were much more active in the production of fibrinogenolysis than were the preparations from hemophilic plasma. Only hemophilic preparation 6 pro-

<sup>1</sup> Presented in part before the American Society for Clinical Investigation, May 4, 1942, Atlantic City, New Jersey.

<sup>2</sup> The expenses of this investigation were defrayed, in part, by a gift to Harvard University from the Smith, Kline & French Laboratories, Philadelphia, Pennsylvania; and, in part, by a grant given "in recognition of Dr. Francis W. Peabody's services to the Foundation" by the Ella Sachs Plotz Foundation.

<sup>3</sup> Graduate Fellow, Belgian American Educational Foundation (1942-1943).

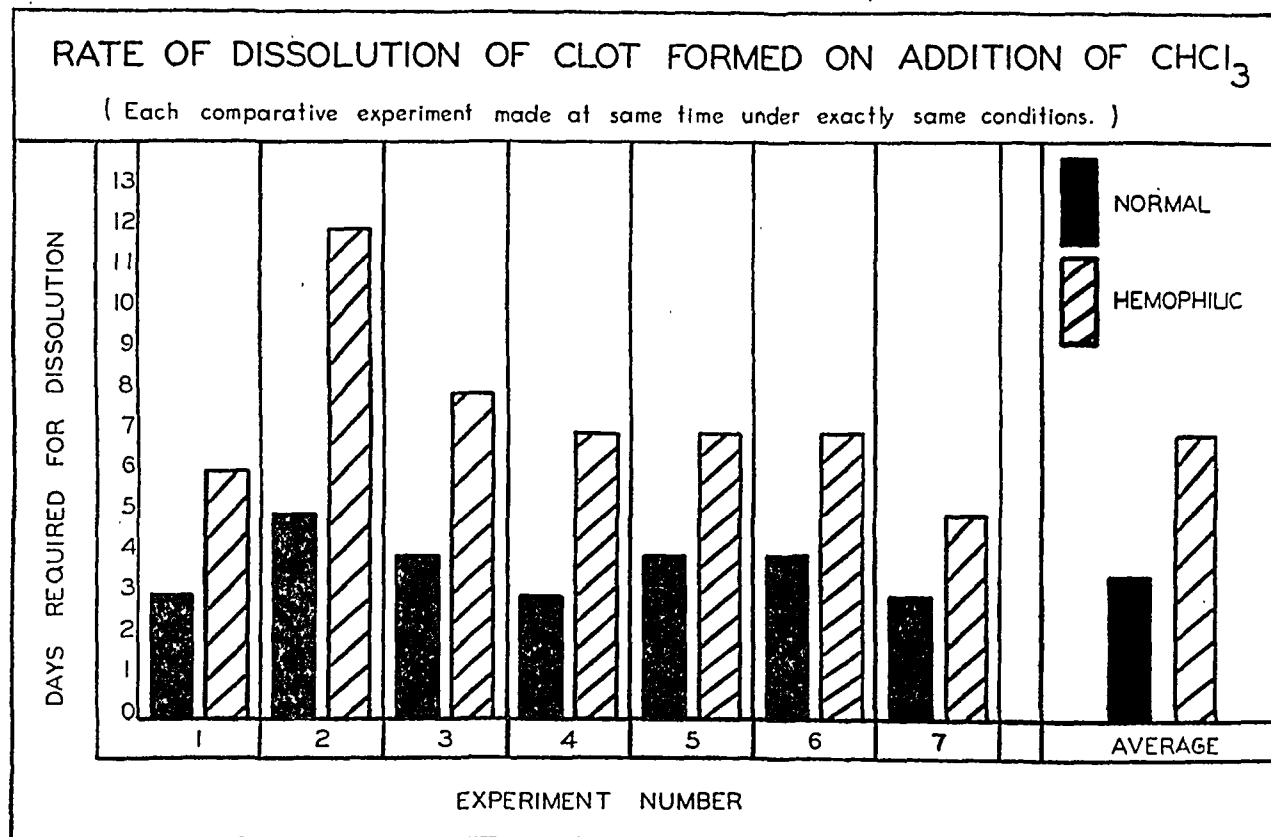


FIG. 1. COMPARISON OF THE PROTEOLYTIC ACTIVITY OF NORMAL AND HEMOPHILIC CHLOROFORM PLASMA PREPARATIONS

Numbers 1 and 2 were platelet-free.

duced an activity in any way comparable to the proteolytic activity of normal plasma preparation. The rate of disappearance of the fibrinogen did not parallel the coagulation time of the hemophilic blood.

TABLE I

*Comparison of the proteolytic activity of normal and hemophilic chloroform plasma preparations*

TIME OF FIBRINOGENOLYSIS		
	Subject	Time for complete Fibrinogenolysis
NORMAL BLOOD PREPARATION	1	10 minutes
	2	12 "
	3	11 "
	4	10 "
	5	10 "
HEMOPHILIC BLOOD PREPARATION	1	more than 60 minutes
	2	more than 60 "
	3	45 "
	4	more than 60 "
	5	50 "
	6	15 "

#### DISCUSSION

The biochemical lesion in hemophilic blood has been the subject of much study. It is generally accepted that prothrombin and fibrinogen appear

to be normal in amount. So far as cell-free plasma is concerned, it appears that the only deficiency at present recognized lies in the clotting activity of the globulin fraction of the serum proteins (3), free from fibrinogen and prothrombin (4).

It has been shown that the addition of "globulin substance" to hemophilic blood, both *in vivo* and *in vitro*, results in a decrease of the blood coagulation time of hemophilic blood toward normal. "Globulin substance" is, however, far from a pure substance. The only chemically pure physiological substance having this coagulation effect is crystalline trypsin (6), the action of which has only been investigated *in vitro*.

The proteolytic enzyme prepared from calcium and cell-free human plasma described by Tagnon and his associates (1, 2, 5) resembles trypsin in many ways. However, the plasma enzyme, unlike trypsin, is a plasma derivative and can be obtained from that portion of the plasma proteins having marked coagulation effect in hemophilia, both *in vivo* and *in vitro*. While the relation of the plasma enzyme to blood coagulation is not fully

understood, its resemblance to trypsin as a proteolytic enzyme and its relation to known coagulation factors would indicate that it plays some important role in blood coagulation. The data presented in this paper offer still further evidence of its essential character in blood coagulation. On the basis of the evidence submitted in this communication, the activity of the plasma proteolytic enzyme is reduced in preparations of hemophilic plasma as compared to entirely similar preparations of normal human plasma. Whether this reduced activity is due to less enzyme being elaborated or to the presence of powerful inhibitor substances in hemophilic blood remains to be investigated.

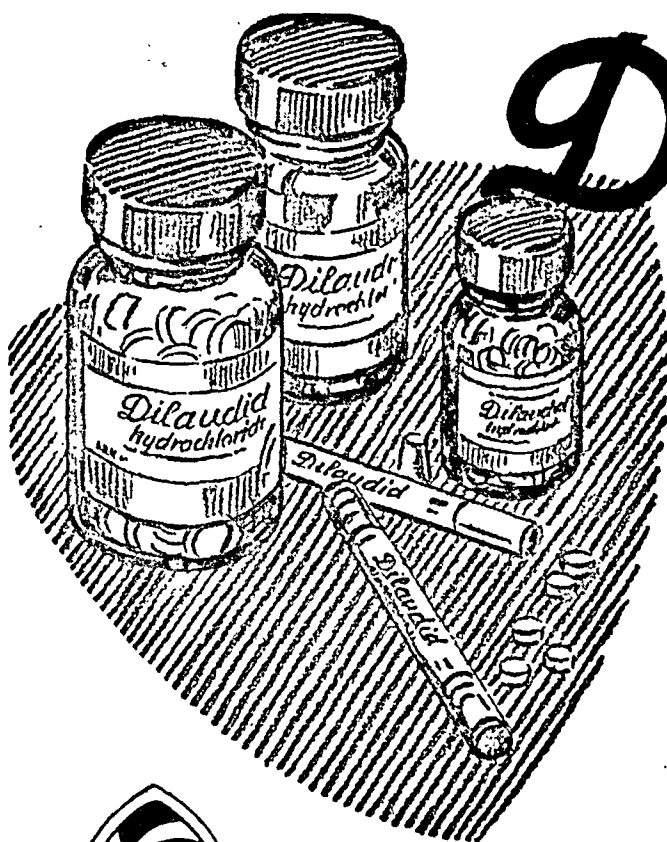
#### CONCLUSIONS

1. The rate of dissolution of the clot obtained by the action of chloroform on hemophilic plasma is much slower than in the case of normal human plasma preparations.
2. The rate at which chloroform plasma preparations derived from normal human plasma can cause fibrinogenolysis is much greater than that of similar preparations from hemophilic plasma.
3. It is concluded that the proteolytic activity

of chloroform preparations of hemophilic plasma is less than for such preparations from normal human plasma.

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W. O. Abbott, H. K. Hartline, J. P. Hervey, F. J. Ingelfinger, A. J. Rawson, and L. Zetzel

Nitrogen Storage Following Intravenous and Oral Administration of Casein Hydrolysate to Infants with Acute Gastro-Intestinal Disturbance.

Alfred T. Shohl

# STUDIES ON THE BACTERICIDAL PROPERTIES OF THE SYNOVIAL FLUID<sup>1</sup>

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(Received for publication June 24, 1942)

Studies on normal synovial fluid and on joint effusions under pathological conditions have been reported (1, 2). In 1919, Labor and von Balogh (3) detected antibodies in the joint cavity during inflammations of the joints. Their results have been confirmed (4 to 6). Positive gonorrheal complement fixations and positive Wassermann reactions in joint fluids have also been reported (1).

The bactericidal activity of various body fluids has been repeatedly investigated (5, 7 to 9), but the only communication of similar studies on synovial fluid was made by Spink and Keefer (10). These authors studied the killing power for gonococci of 44 synovial fluids from 18 patients with gonorrheal and from 13 patients with non-gonorrheal arthritis. Fluids which contained living gonococci and those from patients with non-gonorrheal arthritis were not bactericidal for gonococci. Sterile synovial fluids from patients with gonorrheal arthritis killed the homologous strain of gonococcus. Their bactericidal power was the same or slightly weaker than that of the blood.

According to Collins (11), "the joint fluid in rheumatoid arthritis, on account of its content of polymorphs, is highly bactericidal." Sweetapple (12) pointed out that "synovial fluid is strongly antibacterial while fresh, but excellent pabulum for bacteria when stale." Neither author offered experimental proof for these statements. In rabbits with experimental streptococcal arthritis, synovial fluid obtained more than 3 to 6 weeks after the infection is usually sterile (13).

The object in undertaking the present investigation was to study the bactericidal activity for gram-positive cocci and gram-negative bacteria of the synovial fluid of patients with rheumatoid arthritis and other joint diseases.

It was thought that the detection of a "specific" bactericidal activity for hemolytic streptococci in the synovial effusions from patients with rheumatoid arthritis would support the theory of the streptococcus etiology of this disease. Attempts to isolate a specific microorganism from the synovial fluid in rheumatoid arthritis have been unsuccessful in a high percentage (14), but there is inferential evidence that hemolytic streptococci may be of etiological significance in this disease.

Whenever possible, oxalated blood plasma was tested for its bactericidal activity simultaneously with the joint fluid of the same patient.

In addition, cultures were made and the complement content, as well as the agglutinin titre for streptococci and for *E. coli*, determined. Certain physicochemical and cytological properties of the synovial fluid were also studied.

## MATERIALS AND METHODS

Synovial fluid was obtained by aseptic puncture of the affected joint, usually the knee, of 40 patients with rheumatoid arthritis or other joint diseases.

*Cultures for the bactericidal test.* Ten-fold dilutions (up to 10<sup>9</sup>) of 18-hour broth cultures of a beta hemolytic streptococcus (Strain AB 13) and an *Escherichia coli* were made in broth. The number of organisms in the last dilutions was determined by making pour plates with 0.1 cc. portions of those dilutions. The number of colonies grown in the 10<sup>8</sup> and 10<sup>7</sup> dilutions usually ranged between 20 and 200. These dilutions were used in the experiments. Similar dilutions of overnight broth cultures of 2 other strains of beta hemolytic streptococci, a hemolytic staphylococcus aureus, a Type I pneumococcus, another strain of *E. coli*, and an *Eberthella typhi* (H and O strains) were used in some of the experiments.

*Bactericidal test.* One-half cc. portions of the materials to be tested were mixed in sterile 10 × 100 mm. pyrex glass tubes with 0.1 cc. of suitable, freshly diluted broth cultures of the microorganisms. The tubes were sealed and fastened to a square box on a rotating machine of the type described by Todd (15). After rotation for 24 hours, at 37.5° C., at 8 to 10 r.p.m., 0.3 cc. of the contents of the tubes was plated out with agar, and 0.1 cc. was transferred into tubes of broth. Colony counts were made after 24 and 48 hours. If the rotation time was prolonged to 48 hours, the results did not

<sup>1</sup> Aided by grants from The John and Mary R. Markle Foundation, The Ophthalmological Foundation, and The Dazian Foundation for Medical Research.



change, but if it was reduced to only 6 hours, frequently the microorganisms were not killed.

In about one-half of the experiments, the mixtures were rotated in sterile 20 × 70 mm. vials, closed with a sterile rubber cap through which a sterile injection needle with a sterile cotton plug had been inserted. Sterile tuberculin syringes were used to fill the vials and to withdraw the contents after rotation. The "open" vials offer the following advantages: (1) The mixtures are tested under aerobic conditions, (2) the process of sealing of the tubes is eliminated, and (3) the vials can be used repeatedly.

The results with sealed tubes and open vials were equivalent in all the experiments with *E. coli*. In some experiments with streptococci in broth, however, fewer colonies developed on agar from the "open" vials than from sealed tubes.

*Other examinations of the fluids.* Cultures were made on liquid and solid media. Complement was titrated and agglutinin titres for streptococci and for *E. coli* were determined by the usual methods.

The mucin content was examined by the qualitative "sac and tube" test (1). The relative viscosity was estimated with Ostwald's viscosimeter (16). The specific gravity was determined by the drop method (17). The hydrogen ion concentration at the beginning of the experiment was determined by the bicolor method (18). For cytological studies, total and differential cell counts, and Giemsa and Wright stains, were made.

RESULTS

The bactericidal properties of synovial effusions for hemolytic streptococci and *E. coli*, in relation

to the type of joint disease, are shown in Table I. Thirty-nine fluids were obtained from the knee joint and one from an elbow joint.

Bactericidal properties for hemolytic streptococci were detected in 4 of 37 fluids (10.8 per cent), for *E. coli*, in 34 of 39 fluids (87.1 per cent). Bactericidal properties for hemolytic staphylococci and for Type I pneumococci were absent in 6 fluids from patients with infectious and rheumatoid arthritis, and in 4 fluids from those with non-infectious arthritis. These fluids were not streptococcal.

A strong bactericidal power for *E. typhi* was detected in 10 fluids which also killed *E. coli*.

Simultaneous studies of the bactericidal activity of the synovial fluid and oxalated blood plasma were made with materials from 15 patients.<sup>2</sup> The results are given in Table II.

Three synovial fluids killed streptococci and 6 other effusions were bactericidal for *E. coli*, but the blood of the same patients did not kill streptococci or *E. coli*, respectively. In one instance, the blood plasma, but not the synovial fluid, was bactericidal. There was no difference in the bac-

<sup>2</sup> It should be noted that the bactericidal activities of oxalated blood plasma were compared with those of native synovial fluid.

TABLE I  
Bactericidal properties for hemolytic streptococci and *Escherichia coli* of human synovial fluid obtained from patients with various joint diseases

Lesion of joint	Synovial fluids				
	Number examined	Number which were bactericidal for streptococci	Number which were non-bactericidal for streptococci	Number which were bactericidal for <i>E. coli</i>	Number which were non-bactericidal for <i>E. coli</i>
(A) Infectious and rheumatoid arthritis					
(a) Non-specific infectious.....	2	1	1	2	
(b) Gonorrheal.....	1		1	1	
(c) Syphilitic.....	2		2	2	
(d) Rheumatoid*.....	10	2	7	7	3
Total (A).....	15	3	11	12	3
(B) Non-infectious arthritis					
(e) Intermittent hydroarthrosis.....	2	1	1	2	
(f) Traumatic.....	6		6	5	1
(g) Hypertrophic.....	12		12	11	1
Total (B).....	20	1	19	18	2
(C) Of unknown origin†.....	5		3	4	
Total (A + B + C).....	40	4 (10.8 per cent)	33	34 (87.1 per cent)	5

\* One specimen was not tested for streptococci.  
† Two specimens were not tested for streptococci, a third was not tested for *E. coli*.

TABLE II

Comparison of bactericidal properties for hemolytic streptococci and *Escherichia coli* in synovial fluid and blood plasma of 15 patients with various joint diseases

Lesion of joint	Total number of cases examined	Number of cases with						
		Fluid and plasma bactericidal for streptococcus	Fluid only bactericidal for streptococcus	Fluid and plasma non-bactericidal for streptococcus	Fluid and plasma bactericidal for <i>E. coli</i>	Fluid only bactericidal for <i>E. coli</i>	Plasma only bactericidal for <i>E. coli</i>	Fluid and plasma non-bactericidal for <i>E. coli</i>
Non specific infectious...	1		1		1			
Syphilitic.....	2							
Rheumatoid*.....	6	1	1	2	3	2		
Intermittent hydroarthrosis.....	2		1	3	3	1	1	1
Traumatic.....	1			1	2			
Hypertrophic.....	3			1	1	1		
Total.....	15	1	3	10	7	6	1	1

\* One specimen was not tested for streptococci.

tericidal properties of blood plasma and synovial fluid of 11 patients when tested with streptococci, and of 8, when tested with *E. coli*.

The effect of undiluted and of diluted synovial fluid on increasing concentrations of *E. coli* is presented in Table III.

TABLE III

Effect of dilution of synovial fluid on bactericidal properties for various concentrations of *Escherichia coli*

Dilution of culture	Number of colonies			
	Synovial fluid undiluted	Synovial fluid 1:5	Synovial fluid 1:10	Broth
10 <sup>2</sup>	∞	—	—	∞
10 <sup>4</sup>	1000	∞	—	∞
10 <sup>5</sup>	9	∞	∞	∞
10 <sup>6</sup>	0	0	∞	∞
10 <sup>2</sup> *	90	90	90	90

\* Number of organisms inoculated into 10<sup>6</sup> dilution.  
∞ = innumerable colonies.  
— = no test made.

Five-fold dilutions in broth of synovial fluid did not reduce the killing power for small amounts of *E. coli*. No bactericidal activity was present in ten-fold diluted synovial fluid. With increasing concentrations of *E. coli*, the bactericidal properties of the synovial fluid diminished almost quantitatively. Similar observations were made on the bactericidal activity for streptococci of the few fluids which could be tested.

The influence of inactivation by heat on the killing power of synovial fluid for *E. coli* is given in Table IV.

TABLE IV

Effect of heating of synovial fluid on bactericidal properties for *Escherichia coli*

Medium	Number of colonies after rotation at 37.5° C.		
	Fluid 1	Fluid 2	Fluid 3
Synovial fluid, unheated....	0	0	0
Synovial fluid, 56° C.; 30 minutes.....	0	25	0 (48 hours)
Synovial fluid, 56° C.; 60 minutes.....	—	550	8 (96 hours)
Broth.....	∞	∞	∞

∞ = innumerable colonies.  
— = no test made.

Inactivation in a water bath at 56° C. for 30 minutes did not alter the bactericidal activity for *E. coli* of one fluid, but diminished that of 2 other fluids. Heating at 56° C. for 60 minutes caused a further reduction, but not a complete destruction, of the bactericidal properties of fluid 2. Retardation of bacterial growth by inactivated synovial fluid was observed in specimen 3. Inactivation at 56° C. for 30 minutes completely destroyed the bactericidal activity for streptococci of the few fluids which could be tested.

Eighteen synovial fluids were frozen and kept for 6 months in dry ice at approximately —50° C. The bactericidal activity for *E. coli* was retested frequently. It was found almost unaltered after 180 days of storage. The bactericidal properties of 2 fluids for streptococci were completely lost after storage in dry ice for 3 and 12 days, respectively.

Bacteriological cultures of all fluids used in this study were sterile.

Complement titrations were made on 20 specimens of fresh synovial fluid and of effusions stored in dry ice for periods up to 75 days. The titre of 14 fluids varied between 0.025 and 0.1 cc. (average 0.05 cc.), while that of 6 fluids was above 0.1 cc. The complement titre of stored fluids gradually decreased within 2 months; the bactericidal activity for *E. coli*, however, remained practically unaltered for at least 6 months. The complement titre was very low in 3 of the 5 fluids not bactericidal for *E. coli*. The titre of the other 2 fluids was not tested. The streptococcal fluids had a normal complement titre.

Agglutinins for streptococci were found in 2 synovial fluids in final dilutions of 1:40; these fluids were not streptococcal. No agglutinins for streptococci were detected in the other fluids. The agglutination reactions with *E. coli* were always negative.

The mucin reaction was always positive. The relative viscosity of the fluids varied between 2.9 and 39.1, the specific gravity, between 1.018 and 1.026, and the hydrogen ion concentration at the beginning of the experiment, between pH 7.4 and pH 8.2.

Cytological examinations revealed wide variations in total and differential cell count as reported by previous authors (1). The lowest total cell count was 595 per c.mm., the highest 51,300 per c.mm.

There was no relation between viscosity, specific gravity, hydrogen ion concentration, or cytology of the synovial fluids and their bactericidal properties for hemolytic streptococci or *E. coli*.

#### COMMENT

Most of the synovial fluids from various joint diseases were strongly bactericidal for *E. coli* and *E. typhi*. This activity was closely related to the complement content of the synovial fluid, but heating at 56° C. for 30 to 60 minutes did not completely destroy the bactericidal properties. It has been shown (19) that the third and fourth components of complement require a temperature of 62° to 65° C. for inactivation. No experiments with inactivation at this higher temperature were done in this study. It seems con-

ceivable that the heat-stable components of the complement may be responsible for the bactericidal activity of "inactivated" synovial fluid.

The bactericidal activity for gonococci of gonorrheal synovial fluid was thought to be due to specific antibodies (10). According to Bauer (20), however, "blood from non-gonorrheal patients may show the same variations in bacterial killing power to various gonococcal strains as do patients with acute or chronic gonorrhea." In this study, no agglutinins for *E. coli* or *E. typhi* were found in the fluids which killed these organisms. Bactericidal activity for these microorganisms was detected in the synovial fluid from patients with a variety of joint diseases. It is unlikely that specific antibodies were responsible for this activity.

There is evidence (21) that strains of streptococci vary in susceptibility to bactericidal action of serum and that there is not a good correlation between *in vivo* evidence of streptococcal power of patients' blood and *in vitro* activity of sera. Variations in hydrogen ion concentration and in oxidation-reduction potential play important parts in inhibiting the streptococcal activity of serum (22). No significant variation in susceptibility was noted in the 3 strains used in our experiments, but this does not mean that other strains of greater susceptibility would not have been encountered if a greater number of strains had been tested with synovial fluid, or that wider ranges of hydrogen ion concentration and oxidation-reduction potential might not have brought to light more fluids with streptococcal activity. However, there can be no question that streptococcal activity cannot be as easily and regularly demonstrated as some previous reports indicated (11, 12). Even fluids with high leukocyte counts were devoid of streptococcal activity.

Bactericidal properties for hemolytic streptococci were detected in 2 of 9 synovial fluids from patients with rheumatoid arthritis, and in 2 of 28 fluids from other joint diseases. These results do not justify the conclusion that streptococcal antibodies are usually present in synovial effusions of patients with rheumatoid arthritis, or that their presence is specific for this disease.

From the studies of synovial effusions, it would appear that the bactericidal activities for gram-positive cocci are distinctly different in nature

from those for gram-negative bacteria. The bactericidal activity for gram-positive cocci may be related to the combined action of many phagocytic cells and antibodies. In our studies, evidence for this hypothesis was obtained from a few experiments on the synovial fluid of animals which had received intravenous or intra-articular injections of a sterile irritant or of living or killed hemolytic streptococci (23). All the human synovial fluids examined contained phagocytic cells but only a few effusions were streptococcal. Thus it can be stated that most of the synovial fluids were practically free of streptococcal antibodies. The bactericidal activity for gram-negative bacteria is closely related to the complement content of the synovial fluid and may, therefore, be expected to be present in effusions containing sufficient amounts of complement.

#### SUMMARY

The bactericidal properties of 40 sterile synovial effusions from patients with rheumatoid arthritis or other joint diseases were studied. Four of 37 synovial fluids (10.8 per cent) killed hemolytic streptococci, and 34 of 39 synovial fluids (87.1 per cent) were bactericidal for *E. coli*. The killing power of the synovial fluid usually was stronger than that of the oxalated blood plasma. Bactericidal properties for hemolytic streptococci were detected in only 2 of 9 synovial fluids in rheumatoid arthritis (22.2 per cent), and 2 of 28 synovial fluids in other joint diseases (7.1 per cent).

The bactericidal activity for *E. coli*, but not for hemolytic streptococci, was related to the complement content of the synovial fluid; it was not completely destroyed by heating at 56° C. for 30 to 60 minutes, and remained almost unaltered for 6 months in frozen synovial fluid.

There was no relation between viscosity, specific gravity, hydrogen ion concentration at the beginning of the experiment, or cytology of human synovial effusions and their bactericidal activities for hemolytic streptococci or *Escherichia coli*.

The collaboration of members of the Medical Staff of the New York Hospital, of Drs. H. J. Jaffe and J. E. Blair, and of Miss F. A. Hallman of the Hospital for Joint Diseases in New York City, of Dr. C. Z. Garber of the New York Orthopedic Dispensary and Hospital, and of Dr. J. Freund, Otisville, N. Y., in securing the materials for this study, is gratefully acknowledged.

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# A VISCOUS PROTEIN OBTAINED IN LARGE AMOUNT FROM THE SERUM OF A PATIENT WITH MULTIPLE MYELOMA

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A case of multiple myeloma with hyperproteinemia has recently been observed in which there was obtained, by prolonged dialysis of the blood plasma against distilled water, a large quantity of viscous protein. The excessive quantity of this protein, and the absence of reports of a similar observation in multiple myeloma, with the exception which will be discussed below, makes a record of the circumstances under which it was obtained of interest.

## REPORT OF CASE

A 57-year-old, white, unmarried, female musician commenced to lose weight and grow weak in October of 1941. Later, she developed continuous, dull pains across the back, in both shoulder regions and along the spine. On several occasions, she observed vaginal staining. The familial and past histories were not significant. The menopause had occurred about 7 years previously and was uneventful.

On examination January 12, 1942, waxy pallor was noted. The skin was loose throughout and of poor tone. Edema was not present. On the seventh rib, at about the posterior axillary line, there was a tender swelling approximately the size of a walnut and attached to bluish discolored skin. This mass was not movable and was firm in consistency. The lungs were clear, the heart rate was about 90 per minute, of regular rhythm with an occasional premature contraction. A soft, blowing, systolic murmur was heard over the apex of the heart. The blood pressure was 100/60. No masses were felt on palpation of the abdomen nor was the liver or spleen enlarged. The extremities showed evidences of wasting and the deep reflexes were hypoactive. Pelvic examination did not reveal any infiltration in the parametrium or signs of bleeding.

The patient grew progressively weaker. On January 31, 1942, numerous moist râles were heard over both bases. A firm and deeply attached, somewhat tender prominence had appeared over the fourth dorsal vertebra. Oozing of blood from the gums, and, on several occasions, grossly bloody urine were observed. In addition, during the second week in February, numerous purpuric spots appeared in the skin and larger hematoma over areas of friction. A positive capillary fragility test was observed at the same time. The patient died on March 8, 1942. Roentgen films showed generalized osteoporosis

with punched out areas of rarefaction. A diagnosis of multiple myeloma was made and confirmed by the biopsy obtained just before death from the mass over the rib.

## LABORATORY FINDINGS

On January 13, 1942, the hemoglobin was 4.2 grams per 100 ml. blood; the red cell count was 1,408,000 and the leukocyte count 10,250, of which segmented neutrophils comprised 55 per cent, non-segmented 12 per cent, monocytes 1 per cent, lymphocytes 30 per cent, and metamyelocytes 2 per cent. The blood sugar was 108 mgm., N. P. N. 38 mgm., cholesterol 329 mgm. (of which esters comprised 64 per cent), inorganic phosphorus 4.2 mgm., calcium 12 mgm., and phosphatase 2.2 Bodansky units per 100 ml. blood. The total serum protein was 9.3 grams per cent, of which albumin comprised 3.0 and globulin 6.3 grams per cent. The hematocrit was 16 per cent. The urine was repeatedly negative for Bence-Jones protein.

It was repeatedly noted that immediately after blood was withdrawn by venipuncture, the cellular portion quickly settled to the bottom and the supernatant fluid clotted. This may have been an expression of the pseudo-agglutination mentioned by Magnus-Levy (1) and by von Bonsdorff, Groth and Packalén (2). There was no retraction of the clot, centrifugation being necessary in order to obtain serum.

Prothrombin estimations using snake venom [Shapiro, Sherwin, Redish and Campbell (3)] yielded the following values:

Date	Whole plasma	25 per cent plasma	Difference
January 14	12 seconds	14 seconds	2 seconds (Normal
January 20	18 seconds	20 seconds	2 seconds difference is 10.5 seconds)

Later these were repeated and revealed the following figures, using rabbit brain thromboplastin-calcium chloride mixture (3) instead of snake venom:

Date	Whole plasma	12.5 per cent plasma
February 20	33 seconds <sup>1</sup>	32 seconds
February 27	51 seconds	40 seconds <sup>2</sup>

In both series of estimations, the difference in prothrombin time between whole and diluted plasma is

<sup>1</sup> Normal value for whole plasma is 24 seconds.

<sup>2</sup> This is normal value for this dilution of plasma.

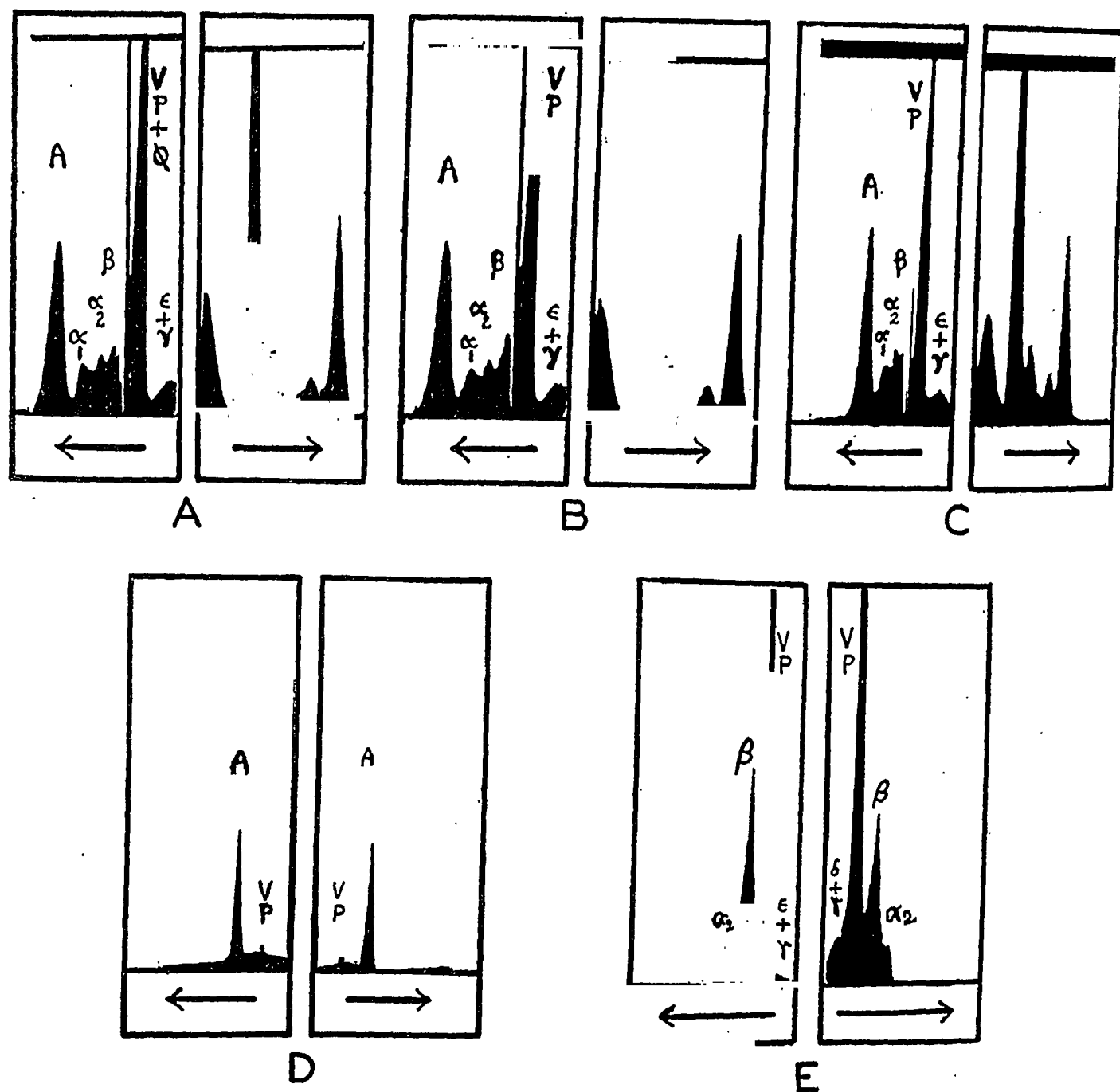


FIG. 1. ELECTROPHORETIC PATTERNS OF THE PATIENT'S PLASMA AND SERUM, OF THE FLUID ABOVE THE VISCOUS LAYER, AND OF THE VISCOUS PRECIPITATE

Pictures of the descending limb of the electrophoresis cell are indicated by arrows pointing to the left; those of the ascending limb by arrows pointing to the right. The sharp boundary seen between  $\beta$  globulin and the VP protein in the patterns of the descending limb is the so-called  $\beta$  anomaly. The Toepler schlieren method with Longworth's scanning modification was used to obtain the patterns. A potential gradient of 8.2 volts per cm. was employed in the barbiturate experiments (Figures 1A, 1B, and 1C) and 3.9 volts per cm. in the phosphate experiments (Figure 1D and 1E).

The current in experiments illustrated in Figures 1A, 1B, and 1C was 12 milliamperes and the conductivities of the fluids were 0.00188, 0.00187, and 0.00199 mhos. The current in the experiment illustrated in Figure 1D was 30 milliamperes and the conductivity 0.00989 mhos. The current in the experiment illustrated in Figure 1E was 14 milliamperes and the conductivity 0.00938 mhos. The duration of the electrophoresis was  $1\frac{1}{2}$  hours in Figures 1A, 1B, and 1D and 1 hour and 1 minute in Figure 1C and 2 hours in Figure 1E. Composition, pH and ionic strengths of the buffers used are given in Table I.

markedly reduced—in fact, in the second series, the plasma diluted to 12.5 per cent yielded a shorter prothrombin time than the whole plasma. This phenomenon, according to the interpretation suggested by Shapiro *et al.* (3), is indicative of an excess of anticoagulant in the blood. These findings are not believed to bear any direct relation to the protein described below. It is noteworthy that the cutaneous hemorrhages were first observed at about the time that the prolongation of the prothrombin time of the whole plasma was observed. This is the subject of another study and will be discussed in a subsequent publication.

The electrophoretic pattern of the blood plasma of this patient is shown in Figure 1A. In addition to the peaks representing albumin, and  $\alpha$ ,  $\beta$ , and  $\gamma$ <sup>2</sup> globulins with mobilities comparable to those given by Moore and Lynn (4) for these components in normal serum, there is a component having a mobility of  $-4.2 \times 10^{-4}$  cm. per second, per volt, per cm. ( $\alpha_2$  globulin in Table I), which is intermediate between the mobilities of  $\alpha$  and  $\beta$  globulins.<sup>4</sup> More striking, however, is the very large peak, marked VP, with a mobility of  $-1.9$ . Figure 1B shows a pattern of the patient's serum. The VP peak is present in apparently undiminished size, proving that the component is not fibrinogen. Furthermore, only 0.5 per cent of fibrinogen was found in the blood plasma. Figure 1C shows the pattern of the blood plasma after removal of the precipitate formed by adding enough solid NaCl to make the concentration 25 per cent. The extra protein is still present. Mobilities, calculated from the descending limbs of these patterns, are given in Table I. Measurements of the area under the VP peak of several patterns show that this component is present to the extent of about 45 per cent of the total serum protein.<sup>5</sup> If one adds the VP protein to the globulins, one obtains an albumin to globulin ratio of 0.34, 0.37, and 0.36 respectively for the three patterns of plasma and serum shown in Figure 1. Omitting the VP from such a calculation yields ratios of 0.92, 1.05, and 0.90.

A solution of the VP protein purified by electrophoresis (in 0.02M phosphate buffer of pH 7.5 and containing 0.15M NaCl;  $\mu = 0.2$ ) became cloudy when heated to 58° in the water bath. At 60° C., it became somewhat

<sup>2</sup> The  $\gamma$ -globulin peak contains also that due to the buffer boundary. Since the ratio of protein concentration to buffer ionic strength is rather high in these analyses of whole plasma and serum, the salt boundary is large. The true  $\gamma$ -globulin peak is somewhat masked by the "false" peak giving erroneous positive mobilities (+0.3, +0.2, +0.2 respectively in Figures 1A, 1B and 1C) for  $\gamma$ -globulin.

<sup>4</sup> See the article by Longsworth, L. G., Chem. Rev., 1942, 30, 323, for evidence of a component separating from albumin in normal human plasma in barbiturate buffer, pH 8.6.

<sup>5</sup> For Figure 1A, VP = 45 per cent, albumin = 26 per cent,  $\alpha_1$  = 6 per cent,  $\alpha_2$  = 6 per cent,  $\beta$  = 11 per cent, and  $\gamma$  = 6 per cent.

TABLE I

Electrophoretic mobilities of the blood proteins, in cm. per second per volt per cm.  $\times 10^{-4}$

Figure	Material in the cell	pH	Al- bumin	$\alpha_1$ Glo- bulin	$\alpha_2$ Glo- bulin	$\beta$ Glo- bulin	VP Pro- tein
1A	Plasma*	7.8	-6.7	-5.1	-4.2	-3.4	-1.9
1B	Serum*	7.8	-6.7	-5.1	-4.0	-3.1	-1.9
1C	NaCl* treated plasma	7.8	-7.0	-5.1	-4.3	-3.5	-1.7
1D	Supernate of viscous protein†	7.4	-5.4	?			-1.9
1E	Viscous protein†	7.3			-3.9	-3.3	-1.9

\* Serum and plasma were diluted 1 to 4 with a buffer consisting of 0.025M lithium diethylbarbiturate, 0.025M diethylbarbituric acid, and 0.025M lithium chloride ( $\mu = 0.05$ ), and dialyzed against 1 liter of the same buffer for 1 day and then against 2 liters the second day. Except for  $\gamma$  globulin, all the components carried a negative charge under these conditions. See Footnote 3.

† Carried out in the same way as serum and plasma experiments but in 0.02M phosphate buffer containing 0.15M NaCl ( $\mu = 0.2$ ). Mobilities calculated from an electrophoretic pattern of the serum in phosphate buffer were: albumin 5.4,  $\alpha$  globulin 3.3, ( $\alpha_1$  and  $\alpha_2$  globulin were unresolved in the descending limb),  $\beta$  globulin 2.5, VP 1.7, and  $\gamma$  globulin 0.1. Mobilities were calculated from the patterns of the descending limb.

viscous. The viscosity increased as the temperature was raised till at 67° C., it was very viscous, and at 72° C., the protein was completely coagulated. When the temperature was raised to 100° C., solution did not occur. It, therefore, was not Bence-Jones protein. The sedimentation constant, measured in the air driven ultracentrifuge (5), was 7.0 S and the diffusion constant  $4.06 \times 10^{-4}$ , corrected to 20° C. The calculated molecular weight is 162,000, thus further excluding Bence-Jones protein. A solution of the protein, containing 1 part in 100,000, yielded a precipitate with rabbit anti-human serum at room temperature. Greater dilutions were not tried.

When the untreated blood plasma was dialyzed in viscose sausage casing against repeated changes of distilled water in the ice box (ca. 2° C.), there formed, after about 10 days, a sharply demarcated layer of liquid at the bottom of the bag which increased in quantity until, after several days, it amounted to about 15 per cent of the total volume. This layer was translucent, had a marked greenish tint, and was so viscous that it was difficult to remove from the bag. In order to determine the relation of this viscous protein material<sup>6</sup> to the VP

<sup>6</sup> By the term "viscous protein," we do not mean that the precipitate is composed of a single molecular species, nor do we use the term to describe the behavior of the precipitate in dilute solution. The term is used to describe the gross appearance of the excessively gelatinous and sticky material. Under identical conditions, plasmas of several hospital patients yielded what appeared to be



peak in the electrophoretic patterns of serum and plasma, a pattern of the fluid above the viscous layer in the dialysis bag was made after removal of some solid protein. The result is shown in Figure 1D. Only a very small amount of the VP protein is present. The large peak seen in the pattern represents albumin. The small peak has the mobility of the VP protein. There is also a small quantity of  $\alpha_1$  globulin (seen more clearly in the original picture). It is believed, therefore, that the viscous protein is derived from the protein represented by the VP peak seen in the serum and plasma patterns. There is reason to believe, however, that they are not identical. For example, when the electrophoretically isolated VP component was dialyzed against water to free it of salts, partial precipitation occurred, but the precipitate was not viscous. Also the electrophoretically prepared protein (mobility  $-1.9$ ) possessed a higher coagulation temperature than the viscous protein.

A pattern of the viscous precipitate,<sup>7</sup> following removal of some water-insoluble protein resting on its surface and repeated washing with water, is shown in Figure 1E. The VP protein is present, together with  $\alpha_2$  and  $\beta$  globulin and possibly some  $\gamma$  globulin. Below we present reasons for suggesting that the viscous protein represents a complex of the VP protein as seen in the patterns of the serum and plasma with another serum protein, or proteins, which forms on removal of salts. If this is so, then the presence of the  $\alpha_2$  and  $\beta$  globulin peaks in the pattern 1E indicate a dissociation of the complex in the presence of the salts comprising the buffer. There is, of course, the possibility that the  $\alpha_2$  and  $\beta$  globulins seen in Figure 1E represent the water-insoluble fractions of these two proteins, co-existing with the VP protein.

When a little of the viscous protein was drawn out into a thread, it had a pearly sheen. It dissolved readily in N/50 HCl and N/50 NaOH and in 0.87 per cent NaCl, yielding limpid solutions. When a solution of the viscous material in saline was heated to 55° C., it became turbid. Raising the temperature to 64° C. precipitated the protein. Further heating to 100° C. did not cause resolution. A solution of the viscous protein gave the usual protein color tests. The Molisch test was positive also. A heavy precipitate resulted on the addition of HNO<sub>3</sub>, trichloroacetic and phosphotungstic acids with potassium ferrocyanide and potassium picrate in the presence of acetic acid. Acetic acid alone did not precipitate the protein. The viscous protein (dried at 100° C. vacuum and P<sub>2</sub>O<sub>5</sub>) contained 13.9 per cent nitrogen. Following hydrolysis with N/1 HCl at 100° C. in a sealed tube for 4 hours, there was found 13.4 per cent<sup>8</sup> reducing substances (as glucose) by the Hagedorn-Jensen method.

on close inspection very small quantities of semi-liquid material along with the usual precipitate of globulin.

<sup>7</sup> This precipitate was obtained by dialysis of a second sample of blood plasma.

<sup>8</sup> This figure is probably higher than the true value owing to the reducing action of certain amino acids formed by hydrolysis.

## DISCUSSION

In multiple myeloma, the extra protein in the serum may differ from case to case. In many instances it is a euglobulin, in some it is one of several kinds of Bence-Jones protein, while in others it has not been classified. Instances of the last are the protein of von Bonsdorff, Groth and Packalén (2), discussed below, and the viscous protein described in this report. Magnus-Levy classified the extra protein in some of his multiple myeloma cases as euglobulin because of its precipitation on dilution of the sera with water. In an extensive study, Gutman *et al.* (6) found that in many cases of hyperglobulinemia in multiple myeloma, the increment consisted of either euglobulin or pseudoglobulin 1, as described by the Howe salting-out method. In the case here described, the excess globulin behaved as pseudoglobulin 2, being precipitated out of the serum on the addition of 30 volumes of 1.5M Na<sub>2</sub>SO<sub>4</sub>.<sup>9</sup>

Multiple myeloma sera may show variations in their electrophoretic patterns also. Longsworth, Shedlovsky, and MacInnes (7) demonstrated a considerably enlarged  $\beta$  globulin peak, while Kekwick's (8) patterns indicate a greatly increased amount of  $\gamma$  globulin in several cases and a large increase in  $\beta_2$  globulin in one instance. (The latter has a mobility between  $\beta$  and  $\gamma$  globulins.) It is interesting that precipitation of the last serum in phosphate buffer pH 8,  $\mu=0.1$ , with 20 per cent Na<sub>2</sub>SO<sub>4</sub>, yielded a sticky product, electrophoretically homogeneous<sup>10</sup> at every pH investigated, but showing four components in the ultracentrifuge. The carbohydrate:N ratio of the globulin so precipitated was 0.167, while that of the globulin prepared from one of the other sera was 0.083. The isoelectric points of the globulins he prepared from the myelomatous sera differed markedly from that of normal human  $\gamma$  globulin, being *ca.* 6.5 for the latter and *ca.* 7.0 for  $\gamma$  globulin in each of 2 of the former, and *ca.* 5.8 for the  $\beta_2$

<sup>9</sup> We are indebted to Dr. A. B. Gutman for this determination. He found the total protein to be 10.5 grams per cent. Of this, the albumin comprised 2.9 grams per cent, and globulin 7.6 grams per cent. The latter was composed of 0.8 per cent pseudoglobulin 1, 6.5 per cent pseudoglobulin 2, and 0.3 per cent euglobulin.

<sup>10</sup> The mobility of the  $\beta_2$  globulin, electrophoretically separated from the corresponding serum, was  $-1.40$  at pH 7.99 phosphate buffer, with  $\mu=0.1$ .

globulin he observed in one of the cases. Gutman and his co-workers found, in a larger series, that the hyperproteinemia in multiple myeloma may be associated with increased quantities of protein possessing the mobility of either  $\beta$  or  $\gamma$  globulin or intermediate between them.

A protein resembling, in certain of its properties, the one we have described was observed by von Bonsdorff, Groth and Packalén in the serum of a patient with multiple myeloma. It was viscous and crystallizable, and it coagulated when its solution was heated to 71° C. It was insoluble in water and physiological salt solution but was soluble in weak alkali and weak acid. Its nitrogen content is given as 13.8 per cent of an incompletely dried sample. No data are given for its carbohydrate content. They report a sedimentation constant  $S_{20} = 7.1 \times 10^{-13}$  and a molecular weight of 200,000. In its viscosity and its solubility in weak alkali and weak acid, and possibly in its nitrogen content, their protein resembles the viscous protein described here. Its insolubility in NaCl solution, its coagulation temperature and serological behavior<sup>11</sup> make it appear different. The serum of their patient also behaved differently from that which we studied in that it (a) clotted at 71° C., (b) separated into two layers after several hours in the ice-box, and (c) deposited crystals of their protein after 48 hours. Our plasma failed to separate into layers even after many weeks in the ice-box; a flocculent sediment formed. We did not observe crystal formation.

A viscous protein has been observed by several investigators in normal human and horse serum. Pieltre (9) identified a viscous protein in normal human serum which he named myxoprotein. Arcaud (10) measured the amount of this protein in normal and pathological body fluids. Doldilhe (11) and Ho Dac An (12) also have reported that a viscous protein separates on dialyzing horse and human sera.

Of considerable interest in regard to the viscous protein herein described is the work of Hewitt (13). This investigator found that, following initial precipitation of protein (euglobulin 1) resulting on dialysis of normal horse and human sera against water, there forms, on extended

dialysis, a precipitate which is semi-liquid, greenish blue in color, and soluble in dilute salt solution (euglobulin 2 in his nomenclature).<sup>12</sup> This description so well fits our viscous protein that it suggests the possibility that the extra protein VP, with the mobility of  $-1.9$ , may react with another serum protein to yield the viscous protein herein described. The differences between the VP protein seen in the patterns of serum and plasma and the viscous protein separating on prolonged dialysis have already been mentioned. In addition, it may be added that Figure 1D shows the absence of  $\beta$  and  $\gamma$  peaks, suggesting that the water soluble portions of these two globulins may have been the ones combining with the VP protein to yield the viscous protein.

#### SUMMARY

A very large amount of viscous protein separated on prolonged dialysis, against distilled water, of the blood plasma from a patient with multiple myeloma. It contained 13.9 per cent nitrogen and, following hydrolysis, yielded 13.4 per cent reducing substance (as glucose). It coagulated at 64° C. Solubility and precipitation characteristics are given.

Electrophoretic examination of the plasma and serum showed a very large peak with a mobility of  $-1.9 \times 10^{-5}$  cm. per second per volt per cm. (barbiturate buffer, pH=7.8,  $\mu=0.05$ ). This material had a molecular weight of 162,000. It coagulated at 72° C. and did not redissolve when the temperature was raised to 100° C. It is neither fibrinogen nor Bence-Jones protein. There is some evidence to suggest that it is a normal serum globulin which reacts with another serum component to yield the viscous protein which settles out on freeing the blood plasma of salts by dialysis.

We wish to thank Miss Helen Sikorski for performing the diffusion and electrophoresis experiments. We are indebted to Dr. J. B. Weiss (Hospital for Joint Diseases) for permission to study this case. We are greatly indebted to Dr. A. E. Severinghaus for the use of his air-driven ultracentrifuge.

<sup>11</sup> See the recent report of further serological examination of their protein: Packalén, T., *Acta Path. et Microbiol. Scandinav.*, 1940, 17, 263.

<sup>12</sup> Hewitt finds that by mixing pseudoglobulin A (by which he designates the fraction of water-soluble globulin precipitated by  $\frac{1}{3}$  saturation with ammonium sulfate) with his "globoglycoid" he obtains a precipitate of euglobulin 2, semiliquid in consistency, which presumably is the same as he obtains on prolonged dialysis of serum.

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# TUBULAR REABSORPTION OF PHOSPHATE IN THE DOG<sup>1,2</sup>

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Harrison and Harrison (1) have recently claimed that "under standard conditions there is a limiting maximal rate of reabsorption of phosphate by the renal tubules which does not vary when the concentration of phosphate in plasma is elevated by the administration of phosphate salts." They believe that this phenomenon is analogous to the limiting maximal rate of tubular reabsorption of glucose and other sugars, first demonstrated by Shannon and Fisher (2). The existence of such a maximal rate of tubular reabsorption has not been demonstrated for any other ion (3, 4), so that, if Harrison and Harrison are correct, the behavior of phosphate is probably unique. Before accepting this conclusion, it seemed wise to study the excretion of phosphate over a wider range of phosphate concentration in serum than did Harrison and Harrison. The experiments reported here also permit certain other observations on the excretion of phosphate.

## MATERIALS AND METHODS

Adult female dogs were used throughout. Creatinine solution, 10 grams per 100 cc., was injected intraperitoneally at the beginning of each experiment. The exact experimental procedure has been previously described (3). Catheterized urine was collected before and in successive periods after the infusion of neutral isotonic sodium phosphate solution. Blood specimens were obtained at the beginning and at the end of each collection period. Mean serum concentrations and clearances were calculated according to the method of Winkler and Parra (5). Inorganic phosphate was determined by the method of Fiske and Subbarow (6), creatinine by a modification of the method of Folin (7). Anaerobic ultrafiltrates of serum were prepared by the method of Lavietes (8). Creatinine clearance is assumed to be identical with glomerular filtration rate in the dog (9).

<sup>1</sup> Part of the material in this paper is drawn from a thesis submitted by Robert W. Ollayos to the Yale University School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Medicine, 1941.  
<sup>2</sup> Aided by grants from the Ella Sachs Plotz Fund, the Emerson Fund, and the Fluid Research Fund of the Yale University School of Medicine.

## RESULTS

The results in four experiments following the injection of isotonic neutral sodium phosphate mixtures are recorded in Table I. The method of calculation is that of Harrison and Harrison (1). Rate of glomerular filtration for each period is calculated by multiplying together creatinine clearance and mean concentration of phosphate in serum. The rate of tubular reabsorption is then measured by the difference between this rate of filtration through the glomeruli and the final rate of excretion in the urine. Obviously, the rate of reabsorption steadily increases as concentration rises, so that over the range studied there is not even a suggestion of any "maximal" rate of reabsorption. This is true in spite of the fact that the serum concentration was raised to much higher levels than those attained in the experiments of Harrison and Harrison. Indeed, two of the four animals later developed tetany, and died within twelve hours following the infusions of phosphate, suggesting that the levels reached in our experiments approached the limits of physiological tolerance. The highest value for tubular reabsorption reported by Harrison and Harrison was about 3.50 mgm. per minute. However, rates of reabsorption three and four times this supposed "maximum" are found in the experiments of Table I.

Serum inorganic phosphate concentrations were used directly in the calculations of the rate of glomerular filtration of phosphate in Table I. Implicit in this procedure is the assumption that the concentration of phosphate in glomerular filtrate is equal to that in serum. The justification of this assumption appears in Table II, in which the concentrations of phosphate in ultrafiltrates of plasma (using cellophane membranes) are compared with the concentrations of phosphate in serum water, in three out of four of the experiments of Table I. Evidently an inconsiderable fraction only of the phosphate of serum is not

TABLE I  
*Intravenous injection of neutral isotonic sodium phosphate solutions*

Experiment number Dog weight	Period*	Duration	Urine flow (1)	Phosphate		Clearance		Amount of phosphate		
				Urine (2)	Serum† (3)	PO <sub>4</sub>	Creatinine (4)	Filtered (3) × (4) 100	Excreted (1) × (2) 100	Reabsorbed (3) × (4) - (1) × (2) 100
kgm.		minutes	cc. per minute	mgm. per cent		cc. per minute		mgm. per minute		
1. 20.5	1	38	0.92	589	14.7	37	77	11.32	5.42	5.90
	2	60	0.60	703	13.4	32	69	9.25	4.23	5.02
	3	85	0.44	740	12.6	26	51	6.43	3.22	3.21
	4	155	0.26	686	10.7	17	28	3.00	1.78	1.22
2. 15.0	1	35	3.72	235	40.0	22	54	21.60	8.73	12.87
	2	68	1.40	291	25.0	16	31	7.75	4.05	3.70
	3	65	0.80	292	18.5	13	25	4.63	2.33	2.30
	4	63	0.78	266	16.6	13	23	3.82	2.08	1.74
3. 13.2	1	26	3.85	391	34.5	44	90	31.00	15.05	15.95
	2	29	2.31	377	21.0	41	87	18.27	8.71	9.56
	3	28	1.43	384	16.8	33	81	13.60	5.49	8.11
	4	57	0.79	429	13.5	25	59	7.96	3.39	4.57
	5	66	0.50	474	10.0	24	70	7.00	2.37	4.63
4. 17.6	1	31	2.87	332	20.0	48	74	14.80	9.54	5.26
	2	21	1.76	276	12.5	39	80	10.00	4.86	5.14
	3	67	1.10	284	10.2	31	60	6.12	3.12	3.00
	4	82	0.65	403	8.5	31	48	4.08	2.62	1.46
	5	30	0.47	479	7.9	28	66	5.21	2.25	2.96
	6	25	0.36	549	7.5	26	68	5.10	1.98	3.12

\* Successive periods after the infusion.

† Interpolated values.

ultrafilterable. Any colloidal complex of calcium and phosphate which might be formed is therefore quantitatively unimportant. If these concentrations in ultrafiltrate are substituted for concentrations in serum in the calculations of Table I, the estimated rates of reabsorption are only slightly altered.

TABLE II  
*Ultrafilterable inorganic phosphate of serum*

Experiment number	Sample	Phosphate		Phosphate, ultrafilterable
		Serum	Ultrafiltrate	
		mgm. per 100 cc. water		per cent
1	1	17.52	17.40	99
	2	14.44	15.55	108
	3	14.60	15.55	107
	4	12.83	12.30	96
	5	10.45	10.48	100
3	1	48.75	44.10	91
	2	19.84	19.28	97
	3	12.92	11.87	92
	4	8.93	7.69	86
4	1	33.95	30.93	93
	2	14.36	13.23	92
	3	9.93	9.24	93
	4	8.62	8.62	100

DISCUSSION

These experiments demonstrate that phosphate is reabsorbed by the tubules in a manner resembling that of other ions, rather than that of glucose and the other sugars. Harrison and Harrison were presumably led to a contrary, and erroneous, conclusion by studying variations in the reabsorption of phosphate over too narrow a range of concentration of phosphate in serum. In addition, the excretion of inorganic phosphate is related to its concentration in serum, and to its simultaneous glomerular filtration rate, in a manner resembling that of other ions. In Figure 1, the excretion rate of inorganic phosphate is plotted against concentration of inorganic phosphate in serum. Data from experiments of Pitts (10), in which hypertonic neutral phosphate solutions were injected, are included for comparative purposes. There is obviously a direct relationship between the two variables, and all the lines except that corresponding to experiment 2 (Table I) fall into the same rather narrow range. No differences are apparent between our experiments with

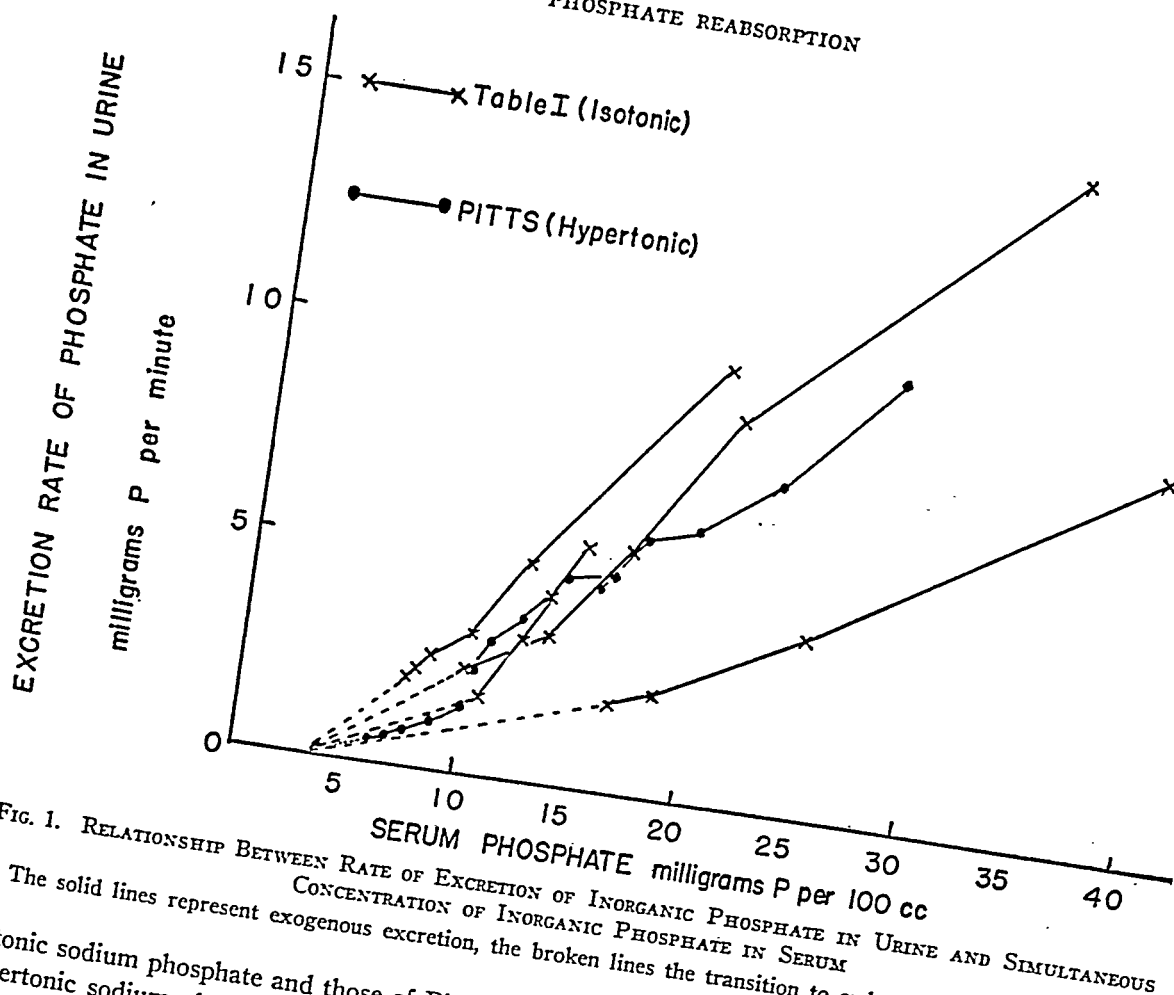


FIG. 1. RELATIONSHIP BETWEEN RATE OF EXCRETION OF INORGANIC PHOSPHATE IN URINE AND SIMULTANEOUS CONCENTRATION OF INORGANIC PHOSPHATE IN SERUM  
The solid lines represent exogenous excretion, the broken lines the transition to endogenous excretion.

isotonic sodium phosphate and those of Pitts with hypertonic sodium phosphate. Adolph (11) and Pitts (10), in discussing experiments with phosphate injection, have previously commented on the tendency of lines relating phosphate excretion to serum concentration to intersect the abscissa well above the origin, near a point approximating normal serum concentration. This is roughly true in our experiments, but on close examination it appears that the upper portion of the lines would not, if prolonged, cut the abscissa exactly at the endogenous point. Instead, the prolongations would intercept the abscissa well above the endogenous region. The lines representing the whole of phosphate excretion are therefore not exactly straight. The lower portions gradually curve as they join the point corresponding to endogenous excretion. This behavior is very similar to that of sulfate (3) and of potassium (4), and indicates that exogenous phosphate, like exogenous

sulfate and potassium, is excreted according to another law than that governing excretion of the same ions at low endogenous levels.

Phosphate clearance is a function of serum concentration, being higher, the greater the serum concentration (Table I). This is a necessary consequence of the fact that the lines of Figure 1 do not pass through the origin, and is a characteristic feature of the clearances of a number of ions (3, 4). There is a rough correlation between phosphate clearance and creatinine clearance in Table I. Their ratio lies between 0.3 and 0.6. This tendency for the ratio to increase with rising serum concentration is less distinct than that of the ratio of phosphate to xylose clearance in the experiments of Pitts (10). The results are not, however, really inconsistent with his. The values for the ratio are also consistent with those obtained by Fay, Behrmann and Buck (12), who have compared phosphate and creatinine clearances directly.

## CONCLUSIONS

(1) No limiting or "maximal" rate of tubular reabsorption of inorganic phosphate in the dog could be demonstrated.

(2) In this respect, excretion of phosphate resembles that of other ions, rather than that of glucose.

(3) The relation of the excretion of phosphate to its concentration in serum and its relation to glomerular filtration are similar in character to those of sulfate and of potassium.

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# URINARY EXCRETION AND SERUM CONCENTRATION OF INORGANIC PHOSPHATE IN MAN<sup>1,2</sup>

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The relationship in man between the concentration of inorganic phosphate<sup>3</sup> in serum and its rate of excretion in urine forms the subject of this paper. A particular attempt has been made to distinguish between those variations in the excretion rate which passively reflect changes in serum P concentration and those which are due to other causes. Two general types of experiments have been employed. In the first type, no P, other than that in the usual diet, was supplied. The concentration of P in serum and its rate of excretion in the urine were compared under a variety of circumstances. Normal diurnal variations, the effects of the ingestion of food and water, sleep, activity, and the injection of insulin, were all considered. In the second group of experiments, the relationship between renal excretion of P and the concentration of P in serum was studied after the latter had been artificially raised by the intravenous infusion of phosphate. Such observations in man have apparently never before been described, except for one observation by Schultz (1), at moderate elevations of the serum P.

## MATERIAL AND METHODS

The experimental subjects included normal individuals and patients with diabetes mellitus. Urine specimens were collected in successive intervals by voluntary voiding, every effort being made to make the collections as complete as possible. Blood specimens under oil were obtained from time to time, allowed to clot, and the serum promptly withdrawn. Inorganic phosphate was determined by the macro method of Fiske and Subbarow (2). Ultrafiltrates of serum were obtained by the

method of Laviertes (3). Diurnal variations of renal excretion were studied in a healthy, young, adult, medical student (R. O.). In most of the experiments, collections were begun about seven in the morning and continued at approximately hourly intervals throughout the day and evening. Blood samples were obtained five or six times during the experiment. The intervals between were somewhat irregular, since some attempt was made to obtain samples in as close a relation as possible to changes in the rate of urinary excretion of P. The subject fasted from the preceding evening until the end of the observation period. Water was given at the rate of 200 cc. per hour during the day of the experiment. The subject remained ambulatory during the period of observation, reading, writing, or doing simple laboratory tasks; no strenuous exertion or exercises were performed. On some days, the customary hours of waking and sleeping were reversed. The subject breakfasted about seven a.m., then fasted for the next twenty-four hours. Collections of urine were started at seven p.m. of the same day and continued throughout the night. The subject slept in the afternoon, and stayed awake all night while urine and blood samples were being collected.

Serum P and renal excretion rate of P were studied in three hospitalized, diabetic patients under insulin therapy. The dietary and therapeutic schedules were not disturbed. Serum P and urinary excretion rate of P were followed in relation to meals and to insulin therapy during the morning and early afternoon, in very much the same way that they were studied in the normal subject.

Four subjects, three normal and one with mild essential hypertension, received infusions of 500 cc. of isotonic neutral sodium phosphate solution. The phosphate solution was made up by dissolving 12.33 grams of  $\text{Na}_2\text{HPO}_4 \times 12 \text{ H}_2\text{O}$  and 1.12 grams of  $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$  in approximately 500 cc. of freshly distilled water. The solution was sterilized by boiling, and brought to exactly 500 cc. volume, by the addition of sterile distilled water, just before giving. A sample was always tested with a phenol red indicator to be certain that its pH was not above 7.3. The infusion was given over a period of 30 to 40 minutes. None of the patients had subjective symptoms of discomfort or objective evidences of reaction during or after the infusion. Numbness, tingling, and tetany were entirely absent. Following the infusion, blood and urine samples were obtained at intervals of forty-five to ninety minutes, the exact intervals being indicated in Table I. The experiments continued for six to eleven hours after the infusion; the subjects received water at

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<sup>3</sup> Hereafter the symbol "P" will represent "inorganic phosphate."



TABLE I  
*Intravenous infusion of neutral isotonic sodium phosphate solution*

Experiment number Body weight	Period	Duration	Urine flow	Concentration of inorganic phosphate		Clearance of inorganic phosphate	Distribution of inorganic phosphate		Remainder
				Urine	Serum†		Recovered from urine	Present in extracellular fluid	
kgm.		minutes	cc. per minute	mgm. per 100 cc.		cc. per minute	per cent of total amount injected		per cent
1. 77.8	1	35	6.23	3		5			
	2	65	2.20	12	3.99	7			
	3	58	2.19	36		21			
	4	157	0.82	55	3.48	13			
	5*	52	3.04	314	17.35				
	6	53	2.49	395	10.80	73	32	48	20
	7	40	1.80	457		84			
	8	45	1.16	464	7.59	63	50	27	23
	9	60	1.09	345	6.00	53	56	20	24
	10	60	1.01	263		43			
	11	60	0.68	301			64		
2. 77.6	1	60	0.73	44	2.52	13			
	2	120	0.79	44		13			
	3	60	1.80	61		38			
	4	120	0.37	12	3.15	14			
	5*	28	4.64	198	18.91				
	6	32	3.72	303	12.51	72	20	61	19
	7	45	3.96	216		70			
	8	40	2.92	167	8.78	51	37	37	26
	9	50	1.88	232	7.08	55	44	26	30
	10	195	0.81	259			55		
3. 86.4	1	60	0.61	65		15			
	2	35	0.56	52	2.69	11			
	3	90	0.35	54		6			
	4	43	1.72	38	3.33	22			
	5*	57	8.98	133	13.43				
	6	42	9.76	118	7.35	105	37	29	34
	7	90	3.31	161	5.46	73	52	15	33
	8	68	1.19	276		63			
	9	120	0.82	275			68		
4. 56.3	1	65	2.38	12	3.19	9			
	2*	69	4.97	167	20.07				
	3	43	2.60	406		79			
	4	37	2.54	251	9.60	55	40	30	30
	5	45	2.82	189	7.08	58	47	18	35
	6	45	2.60	79		28			
	7	75	2.68	45		19			
	8	45	8.00	30	4.98	42	54	8	38
	9	75	3.80	39	4.54	30	56	6	38
	10	65	7.15	16		27			
	11	55	0.80	128	3.71	26	59	2	39
	12	60	0.92	120		29			
	13	120	2.75	40			62		

\* Injection given during this period.

† Sample at end of period.

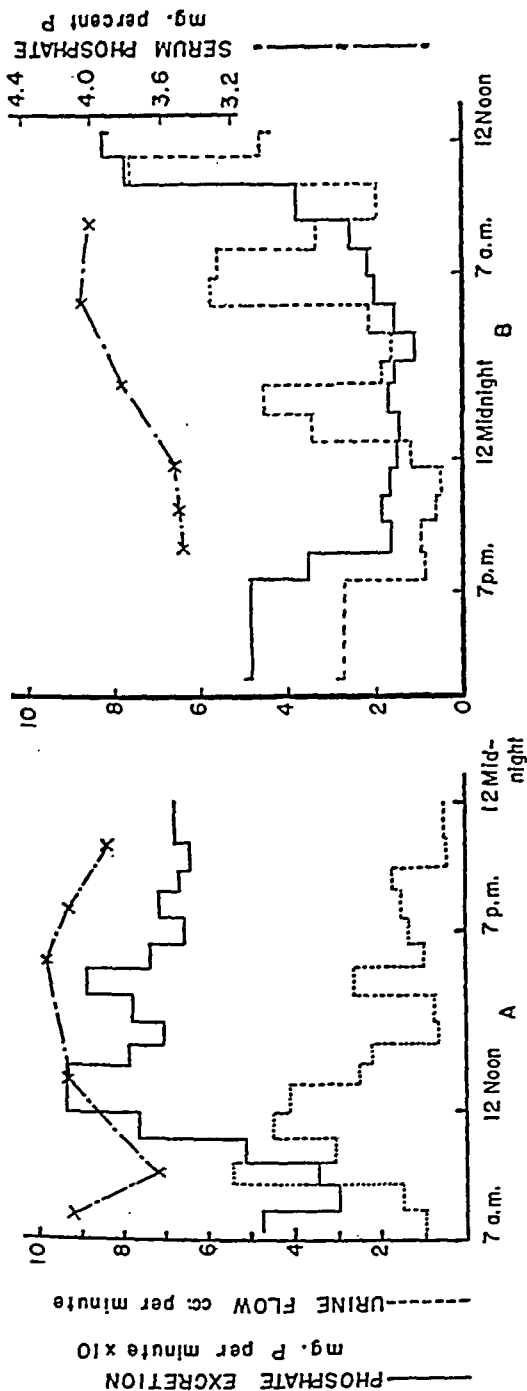
the rate of 200 cc. per hour, but no food. Clearances of P were calculated by a method previously described (4).

## RESULTS

### (A) Excretion in the normal fasting subject

In Figure 1A are presented the diurnal variations of P excretion, and of serum P, in the normal fasting subject, on a single typical day. The excretory rate first fell off sharply during the

second hour after arising, then gradually increased during the late morning hours, reaching a maximum in the early afternoon. In experiments in which the customary rhythm of sleeping and waking was reversed (Figure 1B), the excretory rate fell off sharply in the second hour after the subject had arisen, just as it did in the early morning hours of the control experiments. However, instead of increasing again after 2 or



FIGS. 1A AND 1B. DIURNAL VARIATION OF P EXCRETION AND OF SERUM P IN NORMAL FASTING SUBJECT, (A) WITH CUSTOMARY HOURS OF SLEEPING AND WAKING, AND (B) WITH REVERSAL OF THE USUAL HOURS OF SLEEPING AND WAKING

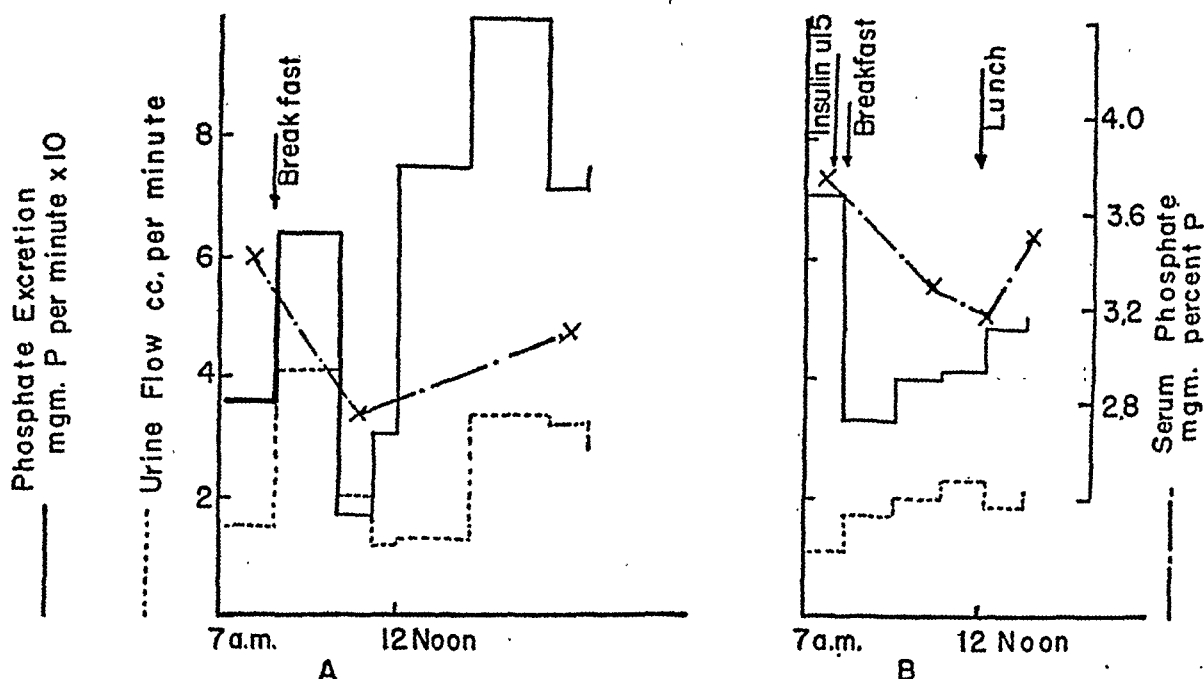
3 hours, the rate of excretion persisted at a low level for another three to nine hours. The transitory increase in the excretory rate of P at about 2 a.m. did not always appear. After falling off for a time, the rate of excretion once again increased markedly during the morning hours after a sleepless night. The characteristic curve of P excretion in this group of observations (Figure 1B), therefore, differs in form from that of the same subject with a normal cycle of sleeping and waking (Figure 1A).

No regular pattern of variation in the serum P, corresponding to variations of P excretion in the urine, can be seen in any of these experiments with the normal diurnal variations of P excretion. In four of the six experiments, the serum P concentration decreased between the first and third hour after rising. In one control observation, there was a sharp, unexplained rise in the serum P to 5.7 mgm. per cent during the early evening, unassociated with any increase in the renal excretion of P.

#### (B) Effects of food and insulin

In Figure 2A, the effects of a high carbohydrate breakfast in a normal subject are shown. No other food was taken during the period of observation. The fall in excretion rate seen in a fasting subject during the second hour after arising is replaced by a moderate rise in excretion rate of P immediately after breakfast. Similar results were obtained in two other subjects. The rate soon falls off again, and the remainder of the daily cycle is indistinguishable from that of the completely fasting subject.

In the three diabetic subjects receiving insulin, there was, on the other hand, an initial decrease both in the serum P and in the urinary excretion rate of P following breakfast and insulin (Figure 2B). No good quantitative relationship and no exact synchrony could, however, be established between serum P and urinary excretion rate of P. Thus, in the experiment of Figure 2B, serum P concentration was still decreasing at the same time that the renal excretion rate was beginning to return to its initial value. In another experiment, there was an almost complete absence of P from the urine, while the concentration of P in serum was 3.1 mgm. per cent, a concentration



FIGS. 2A AND 2B. EXCRETION OF P (A) IN A NORMAL SUBJECT FOLLOWING BREAKFAST, AND (B) IN A DIABETIC SUBJECT FOLLOWING INSULIN AND BREAKFAST

commonly associated with much higher rates of excretion of P in the urine.

(C) *Statistical relationships of urinary excretion of P to serum P concentration and to rate of urine flow*

In Figure 3, the urinary excretion rate of P, in all experiments in which no P was injected, is plotted against corresponding serum concentration. The latter values were obtained by interpolation. There is almost no statistical correlation between the two variables.

In the individual observations of diurnal variations of P excretion, there was no discernable relationship between the excretion rate of P and the rate of urine flow (Figures 1 and 2). This conclusion is confirmed by the evident lack of correlation between these two variables in Figure 4, in which they are plotted directly against one another.

(D) *Effects of infusion of sodium phosphate solution*

The results of four experiments in which neutral isotonic sodium phosphate solution was in-

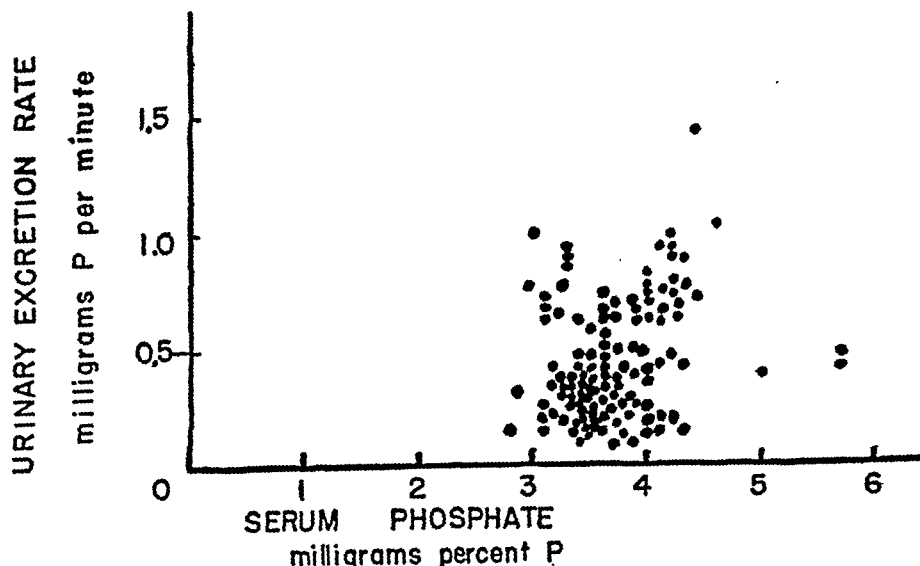


FIG. 3. RELATIONSHIP BETWEEN SERUM P AND URINARY EXCRETION RATE OF P IN ALL EXPERIMENTS IN WHICH NO P WAS INJECTED

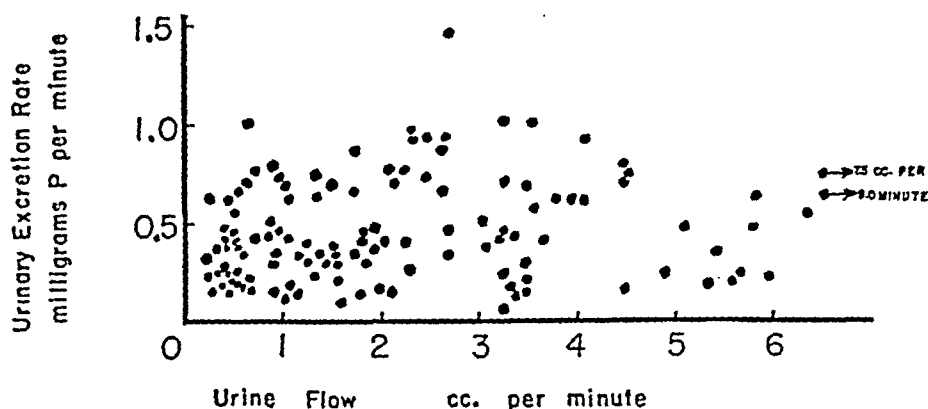


FIG. 4. RELATIONSHIP BETWEEN URINE FLOW AND URINARY EXCRETION RATE OF P IN ALL EXPERIMENTS IN WHICH NO P WAS INJECTED

jected intravenously are given in Table I. Enough phosphate was given to increase the serum P five- or six-fold. The rate of urinary excretion of P was likewise enormously increased, to a greater degree indeed than was the serum concentration. As a result, the clearance of P is several times as great at artificially elevated concentration of serum P than at normal concentrations. As con-

centration declined, the clearance of P also decreased. The exact functional relationship between urine excretion rate and the corresponding mean serum concentration of P is shown in Figure 5. The lines are nearly straight, and tend to intersect the abscissa at or slightly above the region of normal serum concentration. This close interdependence of the two variables contrasts

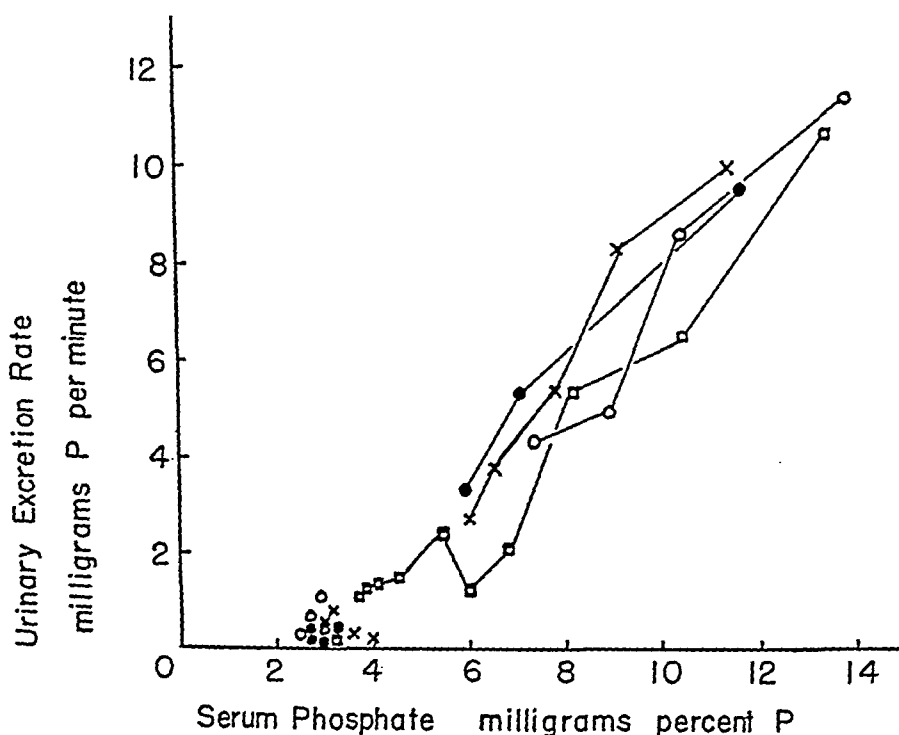


FIG. 5. RELATIONSHIP BETWEEN SERUM P AND URINARY EXCRETION RATE OF P IN FOUR EXPERIMENTS IN WHICH ISOTONIC NEUTRAL SODIUM PHOSPHATE SOLUTION WAS INJECTED INTRAVENOUSLY

sharply with the lack of correlation between the two when no P is injected (Figure 3).

Some estimate of the distribution of injected P between cellular and extracellular fluid of the body has been made in the last three columns of Table I. It has been assumed that the extracellular fluid of the body constitutes 25 per cent of the body weight, and that the concentration of P in the extracellular fluid is identical with that in an ultrafiltrate of serum. Ultrafiltrates of serum were made in one experiment (number 4 of Table I), and the concentrations of P in the ultrafiltrates did not differ by more than 5 per cent from the concentrations in the corresponding whole sera. It was therefore assumed in the other experiments that concentration of P in serum could be used as an approximation of its concentration in ultrafiltrate. The percentage of the injected P present at any time in the extracellular fluid is therefore:

$$\frac{\text{Body weight (kgm.)}}{4}$$

$$\times \text{Increase in serum P} \times 10 \times \frac{100}{2985},$$

where the increase in serum P is expressed in mgm. per cent, and 2985 represents the number of mgm. of P injected in each experiment. Urinary recovery has been estimated by subtracting an assumed average endogenous P excretion rate of 0.5 mgm. per minute from the total urinary excretion of P. The difference between the total amount of P injected, on the one hand, and the sum of the P recovered from the urine and that still present in the extracellular fluid, on the other, must represent P which has entered cells or has been excreted into the gut. Between 20 and 34 per cent of the injected P was regularly present in this "remainder" within the first hour after injection. From that time on, the amount increased very slightly or not at all during the next few hours, while, at the same time, the extracellular fluid was losing its P to the urine. As a result, only 60 to 70 per cent of the injected P was recovered during the course of our experiments, even though in one instance (number 4 of Table I), the serum P had returned to normal.

#### DISCUSSION

Under normal circumstances, the concentration of P in serum evidently does not determine com-

pletely the rate of urinary excretion of P, since the latter may vary quite independently of the serum P (Figure 3). The fact that no constant parallel change in the concentration of P in serum accompanies the wide variation of urinary P during the normal diurnal cycle proves that the rate of urinary excretion is much affected by factors other than serum P concentration (Figures 1A and 1B). On the other hand, we know that, after injection of P, changes in concentration of P in serum are reflected in changes in the rate of urinary excretion of P (Figure 5). It is also quite possible that the same thing is true under more normal circumstances, except that the changes in the serum P are usually masked by quantitatively more important changes, due to other causes. For instance, insulin and carbohydrate administration cause a reduction in the serum P, due mainly to a passage of P out of the extracellular fluid into the cells (Figure 2B); this reduction is regularly paralleled by a reduction in the rate of urinary excretion of P. It seems reasonable to believe that the change in the rate of urinary excretion is simply a reflection of the change in serum concentration. It is possible that, in a somewhat similar way, the marked increase in the rate of excretion of P following prolonged acidosis, due to excessive carbon dioxide (5), only reflects the rise in concentration of serum P. On the other hand, the increased rate of P excretion, which often follows the ingestion of food by normal subjects not receiving insulin (Figure 2A), is apparently unrelated to changes in the serum P. The marked increase in the urinary output of P following parathormone, without much change in the concentration of P in serum (6), indicates that this hormone acts directly on the kidney, rather than indirectly through a control of the serum P. If P in urine is derived from serum P passing through the glomerular filter, then variations in tubular reabsorption must be quite as important, under normal circumstances, as variations in the concentration of P in the glomerular filtrate in governing the rate at which P is finally excreted in the urine.

In marked contrast to the situation at normal concentrations, serum P concentration seems to be the main correlative of the rate of renal excretion of P whenever serum concentration is increased well above normal (Table I and Figure

5). The findings in man in this respect are quite similar to those in the dog (8 to 11) and in the rabbit (7). In view of the different relationship between serum P and renal excretion rate, at normal and at artificially elevated serum P concentrations, the transition from elevated to normal levels of serum P are of especial interest. Experiment 4 of Table I is relevant. At a serum P level between 5 and 6 mgm. per cent, the previously rapid decline in the serum P suddenly slackened. At the same time, the urinary excretion rate became markedly irregular and lost its previous linear mathematical relationship to serum P. This single observation suggests that the shift from a type of excretion closely dependent upon serum concentration to one relatively independent of it may occur fairly rapidly and completely at a critical level of the serum P. In this case, the critical level was a little greater than 5 mgm. per cent. The solitary observation of Schultz (1) indicates a similar fairly abrupt change in the rate of decline of the serum P at 6 mgm. per cent. Urinary excretion was not, however, as clearly dissociated from serum P concentration below this level as in our experiment.

The kidney is evidently the chief channel through which large sudden additions to the P of the body are removed, since about two-thirds of the injected P was recovered from the urine, even before the serum P and the renal excretion rate had returned completely to normal. In addition to the amounts present in the extracellular fluid, there is still a considerable fraction of the injected P which has been segregated elsewhere in the body. The partition between cells (or bone) and the bowel of this segregated balance is not known. As has been pointed out, the segregation takes place almost entirely within the first hour after infusion. After this time, the fraction increases slowly or not at all, so that there can be little continued excretion into the bowel and little loss from the cells. If any considerable portion of this fraction is, in fact, excreted into the gut, such excretion must take place initially with great speed, then suddenly almost cease. Studies with radioactive P have suggested that, contrary to older beliefs, the only P normally excreted into the gut is that which forms a part of the intestinal secretions. Our observations on the distribution of injected P do not indicate any sustained ability

of the gut to adjust its excretion of P to the presence of acute hyperphosphatemia, and are entirely consistent with a negligible increase in excretion into the gut.

The results of the attempt to alter the diurnal P tide by reversing the hours of sleeping and waking are not easily explained in their entirety. The initial fall in the excretory rate of P upon rising in the early evening, after an afternoon's sleep, is similar to that observed in the midmorning hours following a customary sleep during the previous night. This decrease may be related to the increased muscular tone and muscular activity attendant upon waking life (12). The later, long continued reduction of P excretion, with a reversal of normal waking and sleeping hours, has no counterpart in the normal diurnal pattern of P excretion. Had the normal tide been completely reversed, or had the usual excretory pattern persisted, a high rate of excretion of P between midnight and 7 a.m. would be expected. Ingestion of food and recovery from exercise, the commonest means of producing a fall in the renal excretion rate of P, were excluded by the conditions of the experiment. Although no adequate explanation is available, it is apparent that diurnal variations in P excretion are not entirely attributable to the transition from a state of sleep to one of wakefulness.

The changes in serum P concentration and renal excretion rate of P in the diabetic subjects following food and insulin confirm the findings of other workers (13 to 16). The increased renal excretion of P in certain normal subjects following the ingestion of food appears to support the contention that a decrease in the excretion of P does not invariably follow the administration of carbohydrate (17 to 22). The value of this evidence is, however, lessened by the fact that the meals are not purely carbohydrate in composition, and that therefore an unknown amount of fat was ingested along with the carbohydrate.

#### CONCLUSIONS

(1) The rate of urinary excretion of P in man under normal circumstances does not depend primarily on the concentration of P in the serum, although it may be influenced by changes in the latter.

(2) Following the intravenous injection of

large amounts of neutral isotonic sodium phosphate solution in normal man, the rate of urinary excretion of P is mainly determined by the concentration of P in serum.

(3) Injected P is distributed through a greater volume than that of the extracellular fluid.

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# STUDIES WITH RADIOACTIVE DI-AZO DYES. II. THE SYNTHESIS AND PROPERTIES OF RADIOACTIVE DI-BROM TRYPAN BLUE AND RADIOACTIVE DI-BROM EVANS BLUE<sup>1</sup>

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Investigations from these laboratories (1, 2) deal with the accumulation of radioactive di-brom dyes in abscesses and tumors. The radio-dyes are also useful for studies of capillary permeability in so far as their permeation from blood into lymph, too slight to be detected visually in the normal animal, may be quantitatively measured on samples of lymph (3). Dyes of this group make a bond with plasma protein (4), becoming preferentially associated with albumin, and they therefore constitute a radioactive "tracer" for protein under certain experimental conditions.

In view of the interest attaching to such matters, as well as the fact that a large group of chemically related but biologically highly varying compounds—the di-azo dyes—may be made radioactive by this technique, the present paper will describe the method used in rendering the molecule radioactive. Chemical and biological properties of the brominated dyes will also be discussed.

The molecule is made radioactive by the incorporation of two atoms of radioactive bromine in the di-phenyl portion of the molecule (Figure 1). Bromine atoms, so incorporated into an aromatic ring, do not ionize or dissociate. Thus in using the radio-dye for "tracer" experiments, measurements for radio-bromine constitute measurements for the dye molecule as a whole.

The steps involved in this synthesis are as follows:

1. The production of radioactive bromide.
2. Oxidation to radioactive bromine.
3. Bromination of ortho-tolidine.
4. Coupling of this radioactive di-brom tolidine with an amino-naphthol-sulfonic acid to make the finished dye.

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## DETAILS OF SYNTHESIS

### 1. Production of radioactive bromide

We have investigated the production of radio-bromide for this synthesis by three methods: the deuteron bombardment of selenium or sodium bromide, and the neutron bombardment of ethyl bromide. The latter method has been found to be the most satisfactory because it produces large total activities at a high specific activity, both factors being important for such a synthesis as this.

From 20 to 40 kgm. of ethyl bromide<sup>3</sup> are placed by the cyclotron in such a fashion as to secure maximum surface exposure to the neutron beam. The neutron beam, in our experience, has been the secondary result of deuteron bombardment of other elements, beryllium yielding the greatest neutron-intensity. An amount of activity adequate for these purposes lies in the range of 5 to 8 mc.,<sup>4</sup> and will produce a dye which is useful in animal

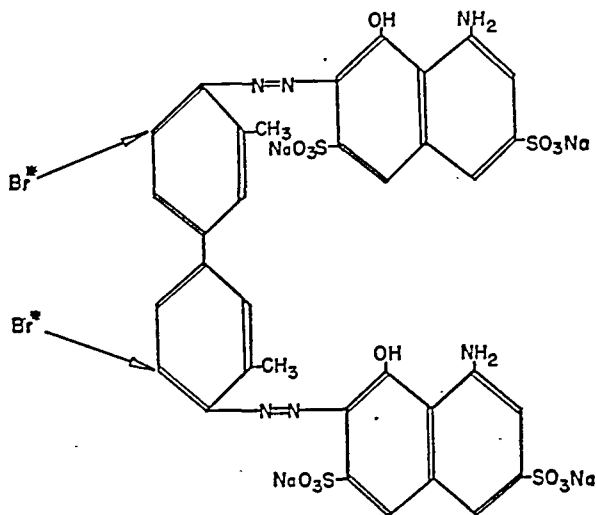


FIG. 1. THE STRUCTURAL FORMULA OF TRYPAN BLUE, SHOWING THE POINTS AT WHICH RADIO-BROMINE ATOMS ARE INCORPORATED INTO THE MOLECULE TO RENDER IT RADIOACTIVE

<sup>3</sup> Other organic bromides such as bromoform or tetrabromomethane, may be used instead of ethyl bromide.

<sup>4</sup> As used here, the term mc. (milli-curie) and  $\mu$ c. (micro-curie) represent an arbitrary correlation with the radiation emitted from a uranium standard. One  $\mu$ c.



experiments for about one week after synthesis. Such an amount of activity may be produced by the deuteron bombardment of beryllium on a probe target, at 250 to 300 micro-amperes, in about 5 hours. Using an external beryllium target as the neutron source, bombardment at 35 to 50 micro-amperes, for about 7 hours, is sufficient. The half-life of  $\text{Br}^{82}$  is 34 hours.

The organic bromide is then extracted in a large separatory funnel with half its volume of water, three extractions being sufficient to secure a large proportion of the extractable radioactivity. The water extract contains the active Br atoms (5, 6). The extract is made alkaline to litmus and then concentrated to a small volume (100 to 200 cc.) by boiling.

## 2. Oxidation to elementary bromine

The concentrated aqueous extract is then transferred to the pot of a small distilling apparatus, the two traps of which are immersed, respectively, in ice-water and in dry ice (in a carbon tetrachloride-chloroform bath). The bromide is oxidized by the dropwise addition of a suspension of  $\text{MnO}_2$  in  $\text{H}_2\text{SO}_4$ , and the resultant bromine condensed and recovered in the two traps, the majority remaining in the first trap, layered beneath water. About 1.0 to 1.5 grams of  $\text{Br}_2$  are distilled, this amount being the result of hydrolysis of the ethyl bromide, the active atoms alone naturally constituting only an extremely small weight of bromine.

## 3. Bromination of ortho-tolidine

One gram of ortho-tolidine (3,3' di-methyl benzidine), recrystallized from toluene or xylene, and dissolved in 75 to 100 cc. of glacial acetic acid, is placed in a 250 cc. three-necked flask. The flask is fitted with a stirring motor, a dropping funnel for the addition of the bromine, and a lead-off to a gas trap.

The radio-bromine is removed from the traps of the distilling apparatus by washing with glacial acetic acid, and this solution is added slowly to the three-necked flask containing the *o*-tolidine solution. The latter immediately turns a dark greenish-purple as the bromine is added. If any bromine beyond that obtained from the distillation is needed to make up a 10 per cent excess, it is now added, and stirring is continued for an hour. The flask is heated to 85° C. in a water-bath to complete the reaction. The resultant suspension is cooled and transferred to a 500 cc. Erlenmeyer flask, and a small amount of  $\text{SnCl}_2$  added slowly, with shaking. This reduces the excess bromine to bromide, and changes the green suspension to a white crystalline precipitate in a clear supernatant. The volume is made up to about 500 cc. with water and the flask is cooled for two hours.

The radioactive di-brom *o*-tolidine (5,5' di-brom, 3,3' di-methyl benzidine) is then collected by filtration, washed with 50 per cent acetic acid, and with water, and then

dried. The product is a grayish powder with a melting point of 195 to 195.5° C., conforming to a previous description in the literature (7), and may be used without further crystallization. The yield is 85 to 90 per cent of the theoretical, on the basis of tolidine.

The precipitation of this product from a large volume of water, and the thorough washing with water, eliminate any radioactivity in the precipitate which might be due to water soluble forms of the isotope, *i.e.* bromide ion.

Successful runs of this bromination have been carried out with as little as 0.15 gram of *o*-tolidine.

## 4. Coupling

The coupling of the radioactive di-brom tolidine with an amino-naphthol-sulfonic acid involves three substituent steps (8).

*a. Hydrochloride formation.* The dried, ground, di-brom tolidine is dissolved in toluene. Dry HCl is bubbled through this solution until no further precipitate forms. This is collected by filtration, washed with toluene and ether, and dried. The yield of amine hydrochloride is 90 per cent of the theoretical.

*b. Diazotization.* All of the hydrochloride is suspended in 150 cc. of water in the presence of four equivalents of HCl, stirred, and cooled to 0 to 15° C. A solution of  $\text{NaNO}_2$ , slightly in excess of the calculated amount (two equivalents), in 10 cc. of water, is run in rapidly. A deep greenish-blue color appears as the diazonium salt is formed. Throughout diazotization, an excess of both HCl and  $\text{NaNO}_2$  should be maintained. The solution is stirred for one-half hour and then about one gram of urea is added to decompose excess  $\text{HNO}_2$ .

*c. Coupling.* In a beaker, a paste is made of the acid to be used for the coupling, consisting of two equivalents in about 50 cc. of water. "H-Acid" (1-amino,8-naphthol, 3,6-di-sulfonic acid) is used for radioactive di-brom trypan blue; "Chicago acid" (1-amino,8-naphthol, 2,4-di-sulfonic acid) is used for radioactive di-brom Evans blue. "Chicago acid" is more water-soluble than "H-Acid" and solution of the latter is affected by adding one equivalent of NaOH in 10 cc. of water, testing with litmus to be sure that the solution is still acid. In computing the weight of acid to be used, account is taken of the fact that the technical grade of the acids is only 88 per cent pure.

The solution is cooled to below 15° C.; 4.0 grams of anhydrous sodium carbonate are added to make the solution alkaline. With vigorous stirring, the diazonium salt is run in rapidly and the solution turns a deep purple. After one-half hour of stirring, the solution is tested for alkalinity and more  $\text{Na}_2\text{CO}_3$  is added if necessary.

At this point, the solution will approximate 200 cc. of a 1.5 per cent solution of radioactive brom-dye. It may be partially purified by dialysis against running tap water. However, it is more desirable to precipitate the dye and make up the solution for injection from pure, dry dye.

This precipitation may be carried out by adding 60 grams of sodium acetate for each 100 cc. of the dye solution, heating to 85° C. and centrifuging while hot. The

of  $\text{Br}^{82}$  by this method gives in the vicinity of  $1.5 \times 10^5$  counts per minute on our Geiger counter, under the geometric conditions we employ.

precipitate is dried overnight at 100° C. and freed of acetate by repeated washings with small amounts of hot 95 per cent alcohol until the washings are acetate-free by test with a drop of  $\text{H}_2\text{SO}_4$ .

A more satisfactory method consists in concentrating the dye-solution by boiling, to a volume of 50 to 100 cc. Absolute alcohol is then added to a final concentration of 90 per cent. The dye is only sparingly soluble in this strength of alcohol and may be centrifuged. This sediment is dried in the oven, the resultant cake is ground up in a mortar and a 1 per cent solution made.

#### COMMENTS

##### 1. Time consumed

In the synthesis of a radioactive compound containing a 34 hour isotope, the time element becomes of prime importance. In our experience, the solution of pure dye, ready for animal injection, may be obtained in 24 to 36 hours from the time the radio-bromide is received from the cyclotron. The dye is then useful for about one week for animal experimentation involving determinations of radioactivity in tissues or body fluids.

##### 2. Radioactivity

The strength of the dry dye at the time it is obtained is in the order of 0.5  $\mu\text{c}$ . per mgm. This is such that 0.00025 mgm. may be measured on the counter, an amount well below the visible range. Higher activities are obtainable with longer bombardments, or by bombarding larger amounts of organic bromide.

Radioactivity measurements may be made on counter or electroscope, the latter instrument being better adapted for the stronger samples. The technic for measurement in tissues or in the intact animal is described in other publications (1, 2). Body fluids containing the dye may be measured directly by drying the sample in a small receptacle which can be placed under the detection instrument in a standard fashion. Care must be taken to use the same amount of fluid for each measurement in a series so as to avoid errors due to self-absorption. For tissue measurements, or where varying amounts of fluid are to be used, a calibration curve may be made to correct for self-absorption.

##### 3. Properties of the dye

If an organic compound is rendered radioactive by the addition of an extraneous element for pur-

poses of biological investigation, it is essential that the radioactive compound have the same biological properties as the non-radioactive compound one wishes to study. In this case, it is important to contrast the chemical and biological behaviour of the brominated dyes with their non-brominated counterparts, trypan blue and Evans blue (T-1824).

*Colloidal di-azo acid dyes* of this group exhibit the following biological properties:

First, slow disappearance from the bloodstream; second, slow or negligible appearance in lymph, urine, and cerebrospinal fluid, under normal conditions; third, accumulation in areas of inflammation and neoplasia; fourth, uptake by the reticulo-endothelial system; and, fifth, staining of certain other cells, such as the tubular epithelium of the kidney.

These biological properties are, in turn, related in some manner to the following physico-chemical properties:

First, the dyes are of a colloidal character in aqueous solution. They are non-diffusible, and congo red, for example, has a particle-size (9) of around 13 Å, considerably larger than one would expect from the molecular weight of around 700. Second, the charge on the colloidal particle is negative (10). In contrast, positively charged dyes, such as Bismarck brown, act quite differently in the organism. Third, they combine with plasma and other body proteins, through acidic or basic attaching groups or adsorption (12). And, fourth, they possess a hydrophobic-hydrophilic molecular configuration of the type shown by Höber (11) to be associated with active transfer in living cells.

*The brominated dyes* herein discussed exhibit the following comparable properties:

1. The brominated dyes are colloidal in aqueous solution, both as evidenced by their relative non-diffusibility through cellophane membranes, and their conduct on attempts at filtration. The rate of diffusion of the dye through cellophane is in the order of 3 to 6 per cent in a 24-hour period. Under the experimental conditions employed, this is the same order of magnitude as trypan blue and Evans blue. When the dye is in concentrations of

1.0 per cent or higher, it is virtually unfiltrable, using ordinary suction filtration apparatus.

2. The charge on the colloidal particle was determined by placing a drop of the dye in an electric field in a simple apparatus such as that described in Burrows (10). The charge on the particle in the case of the two brominated dyes is negative.

3. The dye-protein bond is a matter which has until recently been the subject of little study. Chapman, Greenberg and Schmidt (12) titrated dyes against proteins and showed the union to be chemical (rather than adsorptive) in nature, and stoichochemical in proportion. Rawson's recent work (4) has cast more light on this field. She has kindly carried out tests with our dyes by the Tiselius technique, and by using ultracentrifugation and a cellophane-staining test which she has devised.<sup>5</sup> She finds that the bromo-dyes, like their non-brominated counterparts, migrate preferentially with albumin if the concentration in plasma is in the vicinity of 0.05 per cent or lower. The bromo-dyes are less soluble in water than the non-brominated dyes and stain cellophane to a greater extent.

4. Höber (11) has shown that mono-azo dyes, possessing a structure characterized by a water-soluble "head" and a water-insoluble "tail," have properties in regard to active transfer in living cells not shared by dyes in which both ends of the molecule are either water-soluble or lipid-soluble. Trypan blue and Evans blue possess this structure, the di-amine portion of the molecule being hydrophobic, and the sulfonic-acid-salt, hydrophilic. The brominated dyes likewise share this property, the bromine on the amine rendering it, if anything, less soluble in water.

5. The addition of two bromine atoms produces a dye which is more red than the non-brominated dyes. This is associated with a shift of the peak absorption from 630 m $\mu$ . for Evans blue (13) to 545 m $\mu$ . for di-brom Evans blue, and from 600 m $\mu$ . for trypan blue (13) to 550 m $\mu$ . for di-brom trypan blue. Di-brom Evans blue also shows a color-change with pH at about 8.0. On the alkaline side of this point the dye is quite red, on the acid side, purple. Di-brom trypan blue shows color intensification with addition of small

amounts of either acid or base, with a sharper absorption peak.<sup>6</sup>

6. Dialysis experiments demonstrate that a red component dialyzes through cellophane more rapidly than the purple color comprising the main portion of the dye. This red component is perhaps analogous to the "red impurity" found in trypan blue or unpurified Evans blue (13). This red substance, however, is both colloidal and radioactive and may result from the coupling of the di-brom tolidine with an oxidation-product of the sulfonic acid.

#### SUMMARY

(1) The synthesis of radioactive derivatives of trypan blue and Evans blue is described.

(2) The physico-chemical and biological properties of the brominated dyes are discussed.

(3) A means of studying the *in vivo* conduct of colloids of this group is offered, as well as a means of "tagging" plasma protein.

The authors wish to express their gratitude to Professor Louis F. Fieser for suggestions as to the best mode of synthesizing the dye; to Dr. Baldwin Curtis and the Cyclotron Staff of Harvard University, and to Professor Robley D. Evans and the Cyclotron Group of the Massachusetts Institute of Technology, for providing us with radio-bromine.

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<sup>5</sup> The authors wish to express their thanks to Dr. Rawson for carrying out these studies.

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# STUDIES WITH RADIOACTIVE DI-AZO DYES. III. THE DISTRIBUTION OF RADIOACTIVE DYES IN TUMOR-BEARING MICE<sup>1</sup>

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In a previous communication (1), the concentration of radioactive di-brom trypan blue in animal abscesses was described. The study was undertaken with the underlying concept that such a radioactive substance might provide a method for diagnosing the presence and exact location of inflammatory lesions.

An investigation of the potential usefulness of these radioactive colloids as a means of internally radiating tumors will be described in the present paper. In so far as such usefulness will primarily depend on differential uptake of the radioactive substance by tumor tissue, our initial approach to this subject has been the study of uptake, rather than any attempt to gain a therapeutic effect. The dosage of radioactivity necessary to study tissue uptake of a radioactive substance is considerably below that necessary to gain a therapeutic effect in tumors.

As early as 1909, when Goldmann (2) undertook a study of the biological reactions of certain colloidal dyes, it was noted that tumors appeared to take up the dyes in some concentration. At that time, interest was centered chiefly on histological staining by the "vital stains" (of which the di-azo dyes are examples), and the dyes were administered subcutaneously in large and repeated doses. These circumstances, as is well known, are not conducive to the observation of maximal *selective* staining of any organ or tissue because the animal is so flooded with the dye that all tissues which take it up to any extent show maximal coloration.

Weil (3), Marsh and Simpson (4), and Ludford (5) continued research in this field, and described selective staining of tumor tissue, espe-

cially the periphery of necrotic zones, with trypan blue. Ludford was of the opinion that the dye caused necrosis of the cells which excreted it, and was therefore interested in the possibility of producing regression in the tumors. He observed no such effect, however. Most observers agreed that healthy growing tumor cells did not take up the dye, but that cells of diminished viability or cells in frankly necrotic areas were diffusely stained. In the growing zones of the tumor, only the phagocytic cells of the stroma showed droplets of intracellular dye.

These observations lead to two conclusions about the mechanism of uptake of these dyes which are important for such a study as this. First, that tumors with large amounts of connective tissue stroma might be expected to take up more dye than others, due to the presence of more phagocytes. Secondly that tumor-uptake may be determined by a balance between blood-supply and necrosis in so far as necrotic cells are stained, yet the central areas of necrosis, out of touch with the blood supply, cannot take up the blood-borne colloid.

In 1939, Duran-Reynals (6) revived interest in this subject by showing that under the proper conditions, the tumor may appear by coloration to have taken up the majority of the dye in a highly selective fashion. He was also the first to experiment in tumors with Evans blue, which he found accumulated in the tumors to a greater extent than trypan blue. He observed no therapeutic effect even in large doses, and found maximal selective staining with doses around 0.1 to 0.5 mgm. in mice. Hess (7) corroborated this work and experimented with a number of other dyes.

The concept of treating human tumors with colloidal dyes dates back to the work of Roosen (8) and Bernhardt (9) who injected them into human patients. In 1939, stimulated by interest

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in the more selective concentration described by Duran-Reynals (6) for Evans blue, Brunschwig, Schmitz, and Clarke (10) did a careful study of the accumulation of this dye in human tumors. They reported selective staining of the tumor tissue in 20 out of 30 malignant neoplasms, with non-staining of benign tumors and chronic inflammation. They report no correlation between histological type and ability to localize the dye. Their doses were in the neighborhood of 1 mgm. per kgm. An attempt to increase the permeation of dye into tumor by locally increasing capillary permeability through the use of histamine gave indifferent results; they report very little staining of internal viscera. No therapeutic effect was observed or, indeed, expected, as these authors were aware of the non-toxic nature of Evans blue for tissues.

Since 1940, Zahl and his co-workers (11 to 14) have focussed interest on the possibility of using such dyes as a means of localizing slow neutron-capturing elements (boron, lithium) in tumors and thus radiating the tissue. They used lithiated dyes, derivatives of trypan blue and others of the di-azo acid group. These dyes were evidently made simply by substituting lithium for sodium as the base in the sulfonic-acid-salt, *i.e.* the dyes were lithium salts rather than sodium salts of their respective sulfonic acids. These authors report accumulation of lithium ion alone (injected as lithium chloride) in tumors to the same order of magnitude as the lithium of the lithium-dye complex. This defect in the method probably arises from the fact that the lithiated dye is merely a highly dissociated salt, the dye molecule thus not being a determinant of the lithium distribution. Indeed, the authors find no correlation whatever, after a few hours, between the color-content and the lithium content of the tumor tissue. This is clear evidence in itself that the dye is not determining the distribution of the lithium, but instead the two are assuming distributions of their own. Because of these facts, we have taken care to see that the radioactive isotope in the molecule of the dye is firmly attached to the rest of the molecule and does not dissociate or ionize in solution. Zahl and his group speculate on the usefulness of dyes made radioactive by the inclusion of active isotopes in the molecule, but they do not report work with such dyes.

It is our desire to report, in this paper, work on mouse tumors using radioactive di-azo dyes, made active by the introduction of two atoms of radio-bromine into the molecule. A description of the synthesis is given in a previous publication (15).

#### EXPERIMENTAL PROCEDURE

These studies on the distribution of radioactive di-brom trypan blue and radioactive di-brom Evans blue are based on the injection of these dyes into approximately 65 mice and the measurement of the concentration of the dye in organs and tissues by radioactivity detection methods. The mice used were of various pure strains, C<sub>3</sub>H, C<sub>57</sub>, Strain A and BBC.<sup>3</sup> The tumors studied were spontaneous mammary carcinomas in the C<sub>3</sub>H, and transplanted tumors of a wide variety of types: neurofibrosarcoma, ovarian embryoma (16), rhabdomyosarcoma, and sarcomata induced by carcinogens. The tumors varied widely in size and degree of necrosis. An effort was made to study a variety of tumors with the idea that certain tumors might be found which would accumulate the radioactive colloid to a greater extent than others.

The dose of dye used was in the range of 1 to 5 mgm. per mouse. The radioactivity contained in this amount of dye varied widely from 0.07  $\mu$ c.<sup>4</sup> to 1.2  $\mu$ c. This quantity of radioactivity is undoubtedly below the therapeutic level except for the most sensitive of tumors, and it must be emphasized that our studies were an attempt to study the uptake of the dye rather than to achieve a therapeutic effect. The amount of radioactivity per unit of dye is a function not only of the original peak strength of the radiobromine as produced on the cyclotron but also of the length of time during which the Br<sup>82</sup> has had an opportunity to decay, at the rate of 50 per cent over 34 hours.

The dye was injected intravenously, either in the tail vein or in the femoral vein, in most animals. Other routes of administration were studied but offered no advantages over the intravenous route. The mice were then killed at intervals from 3 to 78 hours. The tissues were excised, weighed, and aliquots, weighing around 0.5 gram, oxidized with concentrated nitric acid in a Coors porcelain ashing capsule, on the steam bath. One-half cc. of acid is used for each 0.5 gram of tissue, and 0.5 cc. of 0.15 M AgNO<sub>3</sub> is added to guard against the loss of activity by volatilization of Br<sub>2</sub> or HBr. The radioactivity in the resultant residue at the bottom of the porcelain capsule is then measured by means of a Geiger counter.

<sup>3</sup> The "BBC" is an inbred descendant of the hybrid BBC mice obtained from Roscoe B. Jackson Laboratories.

<sup>4</sup> The unit  $\mu$ c. (micro-curie) as used here represents an arbitrary correlation with the radiation emitted from a uranium standard. One  $\mu$ c. of Br<sup>82</sup> is defined as that amount giving the same number of counts per minute as a standard amount of uranium under the standard geometrical conditions used on our counter. This amounts to about  $1.5 \times 10^6$  counts per minute.

TABLE I

*Total distribution of radioactive di-brom Evans blue in mouse*  
 Breed of Mouse: Strain A. Weight: 21.5 grams. Dose of dye: 1.25 mgm., totalling 0.297  $\mu$ c. of radioactivity. Duration: 24 hours.

Tissue	Concentration per cent of dose per gram of tissue	Total uptake per cent of dose in entire organ or tissue
Colon	18.6	13.0
Liver	12.5	16.2
Spleen	4.98	1.37
Intestine (small)	4.85	7.30
Stomach	4.81	1.57
Kidneys	4.60	1.72
Tumor*	3.12	2.07
Skin	3.12	11.2
Heart and lungs	2.68	1.39
Testis	1.88	0.70
Brain	0.69	0.28
Bladder	0.26	0.40
Carcass:		
Forelegs and thorax	2.27	5.90
Hind legs	1.72	4.30
Thorax	1.86	3.66
Lumbar region	1.23	2.08
Tail	2.67†	1.87
Head	2.08	4.16
Blood	2.74 per cent per cc.	5.45
Bile	4.63 per cent per cc.	0.46
Urine	2.61 per cent per cc.	1.61
Feces	30.4	15.2
Total		101.9 per cent

\* This tumor was unsuspected at the time of injection; it was a small firm tumor on the diaphragm; every appearance of a slowly growing lesion of low malignancy.

† The tail concentration figure is high because of slight spillage around the tail vein at the time of injection.

This reading is expressed in  $\mu$ c. (micro-curie) or fractions thereof; it is corrected for decay of the  $\text{Br}^{82}$  and for self-absorption according to the amount of tissue in the sample. To make a standard method for expression which will permit comparison between various experiments, the result is expressed as per cent of the injected dose found per gram of tissue, and corrected for the weight of the mouse. This corrected "per cent per gram" figure is used in Tables II, III, and IV.

The full derivation of this figure is as follows:

$$\text{"per cent per gram"} = \frac{\text{Corrected counter reading}}{\text{Weight of sample} - 100} \times \frac{\text{Weight of mouse}}{25}$$

Twenty-five grams is the mean weight of the mice used, and is thus a reference point for weight-corrections resulting in a small correction in most cases. Thus, in a mouse weighing 25 grams, one might expect to find 4 per cent of the injected dose in each gram of the mouse if the radioactivity were evenly distributed throughout the animal. However, about 50 per cent of the dose is found in liver, colon, and feces (see below) in the first 24 hours, and other organs such as kidney and spleen take up differentially high amounts, so that in general it can be stated that any tumor reading of 1.5 per cent per gram, or higher, indicates uptake to a degree greater than one would predict from weight alone. However, as shall be pointed out below, uptake to an extent greater than this does not necessarily indicate differential accumulation of radioactivity to a significant degree; rather, it is the tumor-uptake relative to uptake by other organs which is important.

Using this technique of measurement, the radioactive dye may be measured in body fluids with an accuracy of 2 to 5 per cent, and in tissues with an accuracy of 5 to 8 per cent.

## RESULTS

### *Distribution of dye in animal as a whole*

The total distribution of an injected dose of radioactive di-brom Evans blue 24 hours after injection is shown in Table I. Radioactive di-brom trypan blue adopts the same general distribution save for a somewhat higher concentration in the liver (15 to 20 per cent per gram). The experiment shown in Table I was undertaken as a normal control, and a small tumor on the diaphragm was found at autopsy. The lesion was sufficiently small so that it did not affect the distribution of dye as a whole; the table, therefore, illustrates the distribution of the dye in an essentially normal animal.

It will be noted that liver, colon, intestine, bile, and feces, in combination, take up about 50 per cent of the dose. It is our finding that this repre-

sents the chief excretory route of the dye. Urine figures are low; in this particular animal, the finding of 1.61 per cent of the dose in the urine at 24 hours was rather higher than usual. In contrast to this, the feces had accounted for the excretion of 15.2 per cent of the dose, at a concentration higher than that of any other organ or tissue. The high figures for intestine and colon represent both contents and staining of mucosa by dye. We do not know to what extent dye is excreted through mucosa because dye in the lumen dyes the mucosa and cannot be washed out. Dye present in feces is not visible by its color, due to the dark color of the intestinal content; the color cannot be washed clear of feces as it has stained "fast" the cellulose and proteins of the feces. These facts doubtless explain the fact that past



studies based on color alone have not ascertained the large excretion by this route.

By the end of 24 hours, the blood concentration is quite low as compared with determinations at a shorter interval after injection. Because of this fact, the animals described in the tumor experiments were not killed by bleeding, but the tissues were measured with the blood content in them, the blood concentration altering the tissue reading very little.

The central nervous system shows the least concentration of dye in any tissue and cerebrospinal fluid likewise shows little or no dye, as observed in other experiments in dogs (17). These measurements corroborate previous observations based on color.

#### *Distribution with respect to tumors*

The results of tumor experiments are shown in Tables II and III. Only experiments in which the dye was administered intravenously are included in these tables. All figures are shown in the corrected "per cent per gram" mode of expression, permitting direct comparison between the tumor and tissue concentrations of various mice. Only figures for concentration are given in the tables and not the total uptake; because it is the

concentration of a radioactive substance in tissue which determines the intensity of the radiation to the cells. The relationship of total uptake in tumors to total uptake by other tissues is shown in Figure 1.

The weight of the tumor, in per cent of the animal's body weight, is included in the tables because, in general, any tumor over 7 per cent of the animal's body weight may be expected to show areas of necrosis; any tumor over 15 per cent will have large areas of necrosis which may contain some fluid. There is no observable correlation between size of tumor and acute hemorrhagic necrosis, however. Mice with very large tumors are usually cachectic in appearance and may show anomalous distribution of dye, frequently a low liver concentration suggesting impaired liver function.

The duration of the experiment is indicated in hours from injection of the dye to the death of the animal. In most cases, the animals were killed; a few mice with large tumors died in from 3 to 36 hours and in that case, this interval is considered the duration of the experiment. Mice dying rapidly from too large injections of dye are not included in the protocol.

The ratio of tumor concentration to liver con-

TABLE II  
*Concentration of radioactive di-brom trypan blue in tissues of tumor-bearing mice*

Breed of mouse	Type of tumor†	Size of tumor	Dose	Duration	Concentration of dye					
					Tumor	Liver	Kidney	Spleen	Muscle	Tumor/liver ratio
		<i>per cent</i>	<i>mgm.</i>	<i>hours</i>			<i>per cent per gram*</i>			
C <sub>3</sub> H	SMC	4.7	5.0	3	2.37	16.7	7.67	3.12	3.00	0.14
C <sub>3</sub> H	SMC	8.2	5.0	31	2.80	20.4	9.40	6.50	2.17	0.13
C <sub>3</sub> H	SMC	4.3	3.0	30	2.91	18.1	7.10	7.45	1.07	0.16
Average tumor concentration in SMC = 2.69 per cent per gram. Average tumor/liver ratio = 0.14										
C <sub>3</sub> H	OVT	8.7	2.5	12	3.61	21.2	6.26	5.77	3.94	0.17
C <sub>3</sub> H	OVT	1.0	3.0	27	3.02	19.7	4.32	5.16	1.67	0.15
C <sub>3</sub> H	OVT	10.5	2.5	29	5.06	14.9	7.14	5.57		0.34
C <sub>3</sub> H	OVT	4.8	2.0	31	3.06	12.0	5.75	6.00	3.07	0.25
C <sub>3</sub> H	OVT	1.3	5.0	78	1.46	18.5	6.25	3.34	0.38	0.07
Average tumor concentration in OVT = 3.24 per cent per gram. Average tumor/liver ratio = 0.20										
C <sub>3</sub> H	NFS	8.1	3.0	12	12.7	20.3	19.6	10.9	2.13	0.62

† Tumor types are indicated as follows: SMC, spontaneous mammary carcinoma; OVT, transplanted ovarian embryoma; NFS, transplanted neurofibrosarcoma. The size of the tumor is indicated in per cent of the animal's body weight.

\* Per cent of injected dose per gram of tissue.

TABLE III

*Concentration of radioactive di-brom Evans blue in tissues of tumor-bearing mice*

Breed of mouse	Type of tumor†	Size of tumor	Dose	Duration	Concentration of dye					
					Tumor	Liver	Kidney	Spleen	Muscle	Tumor/liver ratio
		<i>per cent</i>	<i>mgm.</i>	<i>hours</i>			<i>per cent</i>	<i>per gram</i>		
C <sub>3</sub> H	OVT	10.0	2.5	3	2.61	8.71	7.31	5.00		0.30
C <sub>3</sub> H	OVT	11.0	5.0	19	6.15	6.46	5.10	3.12		0.95
C <sub>3</sub> H	OVT	10.0	5.0	19	2.92	7.20	8.10	7.86		0.40
C <sub>3</sub> H	OVT	12.7	2.5	21	2.82	11.6	1.16	4.16	1.00	0.24
C <sub>3</sub> H	OVT	1.8	2.5	21	2.82	11.6	1.16	4.16	1.00	0.24
C <sub>3</sub> H	OVT	10.0	2.75	24	2.37	5.33	8.20	3.19	0.43	0.44
C <sub>3</sub> H	OVT	10.7	2.5	24	4.22	7.95	10.1	8.10	0.50	0.53
Average concentration in OVT = 3.42 per cent per gram. Average tumor/liver ratio = 0.44										
C <sub>3</sub> H	NFS	5.7	5.0	3	0.85	9.06	6.40	3.58		0.09
C <sub>3</sub> H	NFS	4.0	5.0	3	1.26	11.3	10.3	4.0		0.11
C <sub>3</sub> H	NFS	2.0	5.0	3	0.46	14.0	2.5	1.55		0.03
C <sub>3</sub> H	NFS	14.0	3.0	7.5	2.12	9.85	8.70	2.88	2.16	0.46
C <sub>3</sub> H	NFS	7.5	5.0	12	3.50	4.15	4.31	1.16	0.44	0.84
C <sub>3</sub> H	NFS	9.3	2.8	19	6.70	6.90	6.75	2.64		0.97
C <sub>3</sub> H	NFS	8.6	5.0	19	6.30	2.92	7.91	6.25		2.16
Average concentration in NFS = 3.13 per cent per gram. Average tumor/liver ratio 0.66 Average excluding 3-hour experiments = 4.65 per cent per gram. Average tumor/liver ratio = 1.22										
C <sub>3</sub> H	RMS	10.5	3.2	12	3.40	5.60	5.30	1.97	0.715	0.60
C <sub>3</sub> H	RMS	8.0	2.5	12	2.46	7.91	4.20	2.14	1.74	0.31
C <sub>3</sub> H	RMS	2.5	2.5	12	2.64	7.00	11.0	4.20	1.56	0.37
C <sub>3</sub> H	RMS	29.2	2.5	24	1.80	4.72	5.10	2.56	0.74	0.38
C <sub>3</sub> H	RMS	16.0	2.5	25	1.52	4.67	2.92	1.92	0.44	0.32
C <sub>3</sub> H	RMS	17.0	2.5	29	2.21	7.70	6.36		0.791	0.27
Average concentration in RMS = 2.34 per cent per gram. Average tumor/liver ratio = 0.38										
BBC	TMC	17.0	2.5	24	1.66	4.02	4.04	2.91	1.03	0.41
BBC	TMC	14.0	2.5	24	2.26	7.12	9.84	4.30	1.08	0.32
BBC	TMC	10.0	1.5	25	1.55	10.3	9.90	4.76	0.63	0.15
Average concentration in TMC = 1.82 per cent per gram. Average tumor/liver ratio = 0.29										
C <sub>3</sub> H	SMC	3.8	3.0	3	2.10	5.12	4.86	3.08	0.68	0.41
C <sub>3</sub> H	SMC	3.4	5.0	12	2.46	6.10	7.35	3.54	0.86	0.40
C <sub>3</sub> H	SMC	35.0	3.5	24	1.09					
C <sub>3</sub> H	SMC	8.1	2.75	24	3.38	5.31	8.50	3.84	0.70	0.63
Average concentration in SMC = 2.26 per cent per gram. Average tumor/liver ratio = 0.48										
C-57	MCAS	2.8	5.0	6	5.60	9.23	8.80	3.83	0.46	0.61
C-57	MCAS	14.0	3.5	8	3.06	10.0	6.70	2.84	0.94	0.30

Average concentration in MCAS = 4.33 per cent per gram. Average tumor/liver ratio = 0.45

† Tumor types are designated as follows: OVT, transplanted ovarian embryoma; NFS, transplanted neurofibrosarcoma; RMS, transplanted rhabdomyosarcoma; TMC, transplanted mammary carcinoma; SMC, spontaneous mammary carcinoma; MCAS, methyl-cholanthrene induced sarcoma. The size of the tumor is indicated in per cent of the animal's body weight.

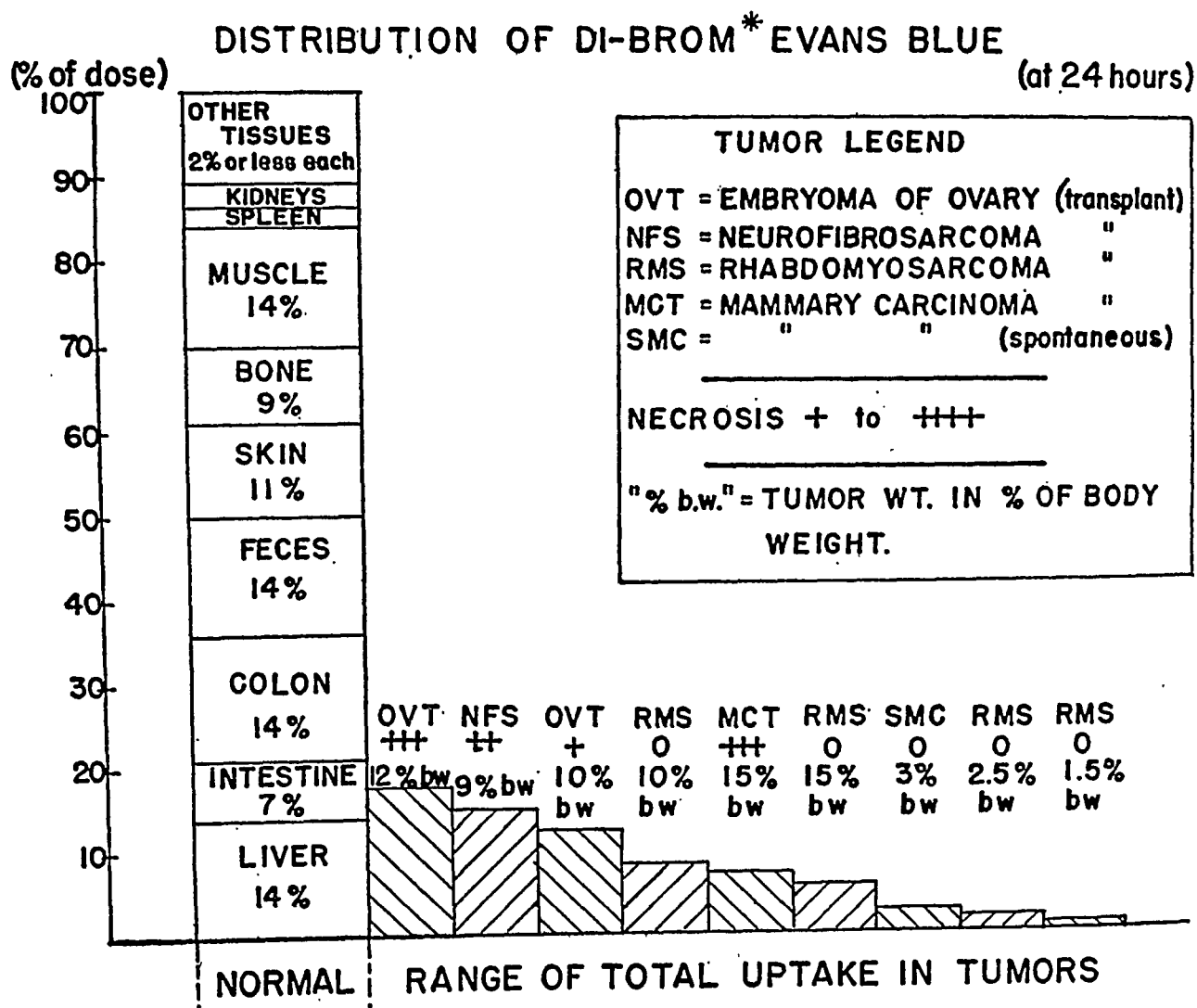


FIG. 1. TOTAL DISTRIBUTION OF DYE AT 24 HOURS, WITH TOTAL UPTAKE OF A REPRESENTATIVE GROUP OF TUMORS FOR COMPARISON

centration is an index of the selectivity of uptake. As will be observed in the table, the relationship between the two is such that were the animal to receive therapeutic radiation by this method, the liver would receive a large dose of radiation and constitute the chief by-effect of the treatment. Any tumor approaching a tumor-liver ratio of 1.0 we consider to have taken up an exceptionally large amount of dye. It will be noted that the two dyes show about the same range of uptake in tumors. There is considerable variation but most of the tumors fall into the 1.5 to 3.5 per cent per gram range, and it can therefore be concluded that the tumor tissue does not actually take up dye to a high order of selectivity. However, the tumor/liver ratios are lower on the average for di-brom trypan blue because of the higher liver concentrations. This indicates greater differential accumulation in tumors for the Evans blue deriva-

tive, making it theoretically the more suitable of the two for internal radiation by this method.

This difference between the two dyes, originally suggested by Duran-Reynals (6), together with the higher liver uptake by di-brom trypan blue (which may be correlated with the faster disappearance rate of trypan blue from plasma observed by Gregersen and Gibson (18)), serves to indicate the delicate adjustment which holds between the biological behaviour of these dyes and their chemical structure. The two dyes in question are isomeric, the only difference being the position of the sulfonate radicals on the naphthalene rings. Gusberg, Zamecnik, and Aub (19) report a similar striking difference in distribution between two organic di-selenides, one with and one without mercury in the molecule.

These facts hold out considerable hope that further investigation of radioactive derivatives of

closely related dyes of the same group may reveal a dye possessing a chemical structure which is correlated with a much higher differential uptake by tumor tissue, and therefore more suitable as a carrier of radiation to tumors. Further research should be undertaken in this direction.

#### *Distribution within tumors*

With this technique, it is also possible to measure the amount of radioactivity appearing in various zones within the tumor. If the tumor is undergoing caseous necrosis in the central area, considerably less dye is found to have permeated into this zone. The same is true if the necrosis has gone on to clear or straw-colored fluid. If, however, the tumor is undergoing necrosis of an acute type, with hemorrhagic fluid in the central area, this zone tends to "pool" the dye from the plasma, and a higher concentration may be found there than in the surrounding tissue. These findings are summarized in Table IV.

TABLE IV  
*Distribution of radioactive dye within necrotic tumors*

Breed of mouse	Type of tumor	Size of tumor per cent	Concentration of radioactive dye				
			Chronic caseous necrosis			Acute hemorrhagic necrosis	
			Wall	Necrotic zone	Fluid	Wall	Necrotic zone
BBC	TMC	17	1.66	0.88			
C <sub>3</sub> H	SMC	35	1.09	0.75			
C <sub>3</sub> H	RMS	29	1.50	1.13	0.18		
C <sub>3</sub> H	RMS	16	1.52	1.11			
C <sub>3</sub> H	OVT	13	2.82	1.70			
C <sub>3</sub> H	NFS	6	0.85				
C <sub>3</sub> H	NFS	4	1.26				
C <sub>3</sub> H	RMS	8			0.32		
C <sub>3</sub> H	RMS	8			0.35		
BBC	TMC	14			0.97	12.7	
C <sub>3</sub> H	OVT	13			0.98	2.46	
					1.75		
					1.76		
							2.26
							2.37

Designation of tumor types as in previous tables. It will be noted that acutely hemorrhagic tissue or fluid accumulates more dye than the surrounding wall, whereas the reverse is true of chronic caseous necrosis.

These variations in dye concentration produced by necrosis are the end-result of that balance between necrosis and blood supply previously mentioned as the conditioning factor in the accumulation of colloidal dyes in tumors. Chronic necrosis of the type which accumulates clear or slightly colored fluid is the result of long-continued tissue ischemia and as such cannot accumulate blood-borne substances such as the radioactive dye. Acute hemorrhagic necrosis, on

the other hand, represents an over-abundance of blood, accompanied by sufficient tissue-destruction to produce increased permeability of the capillaries of the tumor. Such conditions are of course ideal for the accumulation of a blood-borne colloid which leaves the vascular tree at points of increased permeability.

#### DISCUSSION

It is clear that a technique such as this, which can measure the radioactive dye present in tissues, casts some doubt on previous enthusiastic assertions that tumor uptake is highly selective. The latter observations are influenced by the fact that tumor tissue is light or white in color, with the result that dye in small quantities will appear to have stained it markedly, whereas the reverse is true of kidney, liver, spleen, and feces. Of past workers with the dyes, Hess (7) most closely approximated these results. He stated that with careful microscopic methods he never observed a tumor which had more dye in it than the liver, no matter how selective the gross staining appeared to be.

The significant fact remains, however, that the radioactive colloid permeates into and therefore radiates tumor tissue wherever this tissue may be, and no matter how widespread the metastases are. This fact might render such colloids clinically useful, especially if some way offered itself to increase the uptake of dye by tumor.

A means for increasing this uptake is suggested by our results with acutely hemorrhagic tumors. In addition to administering the radioactive dye, coincident therapy with agents increasing capillary permeability or inducing hemorrhage in the tumor might increase the dye-uptake to the point where clinical application would be feasible. Substances producing an increase in capillary permeability such as bacterial products (20, 21), spreading factor (22), histamine (10), or external radiation, might be useful in this regard and justify further study.

#### SUMMARY

1. Experiments are described in which radioactive di-brom trypan blue and radioactive di-brom Evans blue have been injected into tumor-bearing mice.
2. Subsequent quantitative measurement of the

distribution of the radioactive colloidal dye demonstrates a widespread gradual uptake from the bloodstream by many tissues and organs. Large amounts of dye are excreted in the bile and feces in the first 24 hours following injection.

3. In the face of this normally widespread distribution, the uptake of dye by tumors does not appear as selective as when judged by tinctorial methods alone.

4. Possible therapeutic effectiveness of a radioactive colloid would be increased by agents producing tumor necrosis.

5. The ideal radioactive colloid for tumor treatment would be one taken up selectively by tumor tissue to a greater degree than those here reported.

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# AMINO ACID STUDIES. II. PLASMA AMINO ACID RETENTION, AS EVIDENCE OF IMPAIRED LIVER FUNCTION. INVESTIGATIONS IN CHILDREN WITH NEPHROSIS AND LIVER DISEASE<sup>1</sup>

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Kirk (1) reviewed the literature concerning liver dysfunction in relation to amino acid metabolism following the ingestion of glycine or mixtures of amino acids, and, in addition, reported his own experiments. Using the nitrous acid method of Van Slyke, he determined the amino acid N concentration in plasma, before and after the ingestion of 25 grams of glycine, in patients with hepatitis, liver cirrhosis, and obstructive jaundice. He concluded that no definite reduction in the deamination of the amino acids was demonstrable in patients with liver disease and that the glycine tolerance test under the conditions of his experiments was not suitable for the diagnosis of liver dysfunction. He also studied the urinary excretion of amino acids before and after the ingestion of glycine and reported that essentially the same amino acid excretion was observed in normal individuals as in patients with liver disease. Kitamura (2) demonstrated a retention of plasma amino acids following an intravenous injection of certain amino acids in rabbits with livers damaged by chloroform and similar poisons. From the plasma amino acid curves obtained after the intravenous injection of glycine in patients with liver disease, Horejsi (3) stated that little information could be gained by such methods unless severe liver destruction was present. Weicker (4) studied the behavior of blood amino acid levels following the ingestion of a mixture of equal parts by weight of glycine, leucine, asparagine, and tyrosine. He found that in some of the patients with liver disease, the retention of whole blood amino acids persisted longer but that the maximum values obtained were not higher than those obtained in normal controls.

Weech (5) found a progressive fall in the ability of the liver to excrete bilirubin, early in the course of a dog fed a protein deficient diet, and, at autopsy, in some dogs similarly fed, histological evidence of liver damage. Elman (6) confirmed and elaborated on these findings, presenting evidence that morphological damage paralleled liver dysfunction as determined by the excretion of iso-iodeikon. Since liver damage occurs in the hypoproteinemic dog, this experimental animal provided the basis for study in this laboratory (7) of the relation between liver impairment and the clearance of amino acids from the plasma. Retention<sup>2</sup> of plasma amino acids following an intravenous injection of casein hydrolysate was observed in the hypoproteinemic dog early in the course of deproteinization, as shown in Figure 1, and the plasma amino acid retention increased as hypoproteinemia advanced. Evidence that this behavior was probably caused by delay in the rate of deamination was cited.

In nephrosis, Farr and MacFadyen (8) found chronic hypoaminoacidemia and at times, with or without peritoneal infection, a sharp critical drop in the level of plasma amino acid. Also, in nephrosis, it is generally conceded that a defect in the synthesis of plasma protein exists.

The present paper reports investigations of the reactions of nephrotic children to injections of casein hydrolysate and the rate at which the materials were metabolized. In addition, similar studies were carried out on patients with liver disease and normal renal function, and conversely, on patients with kidney disease and unimpaired liver function, in an attempt to evaluate if possible the

<sup>1</sup> Aided by funds from the Emeny Gift and from the Williams-Waterman Fund of the Research Corporation.

<sup>2</sup> Retention will be used throughout the paper to denote increased plasma values of amino acid N above the highest value found in the controls at any time interval following the injection of casein hydrolysate.

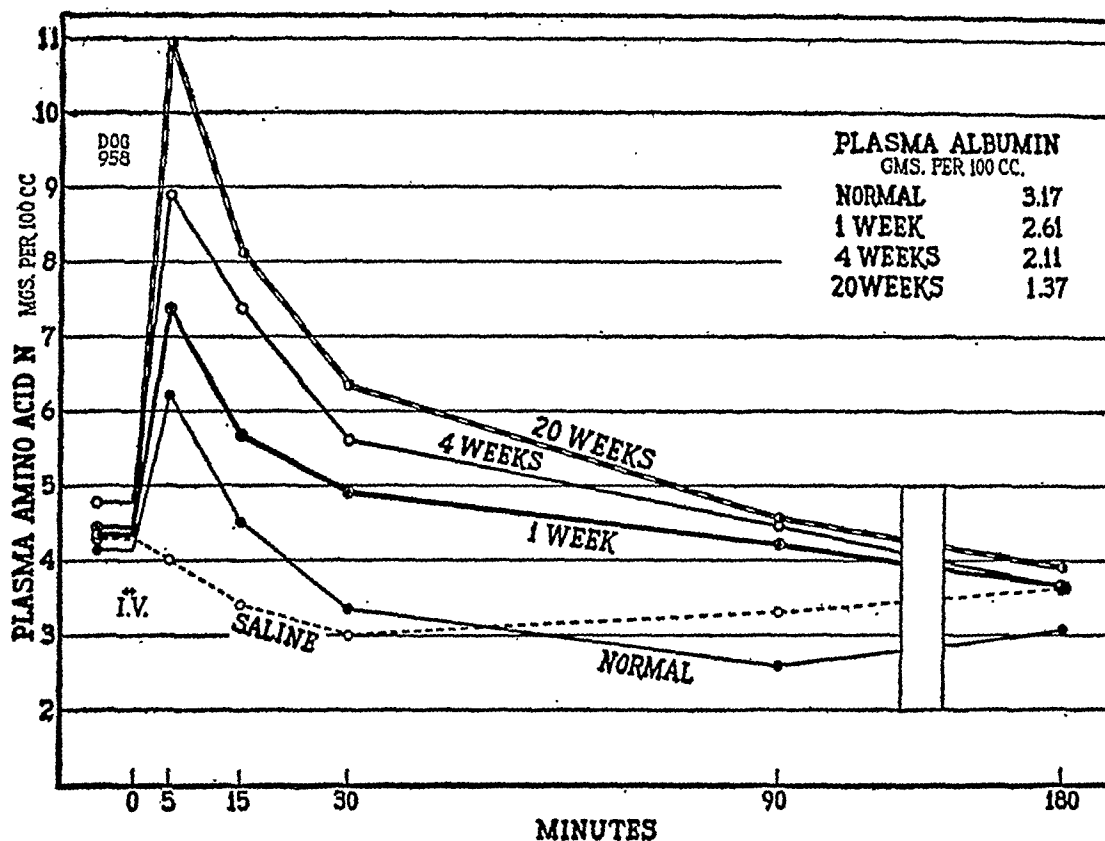


FIG. 1. THE PROGRESSIVE RETENTION OF PLASMA AMINO ACID N AFTER INTRAVENOUS INJECTIONS OF CASEIN HYDROLYSATE IN THE DOG AS HYPOPROTEINEMIA PROGRESSES

The curve marked "normal" represents the reaction while on a normal diet. The curve marked "saline" represents the reaction to a control intravenous injection of 5 per cent glucose in saline, with a normal diet.

part played by each of these important organs in the deamination and disposition of amino acids.

#### PROCEDURE

Amino acid loading curves were carried out in normal controls and in the patients selected for study. In the earlier experiments on children 8 to 10 years of age, 40 cc. of 10 per cent solution of casein hydrolysate,<sup>3</sup> diluted with 40 cc. of 5 per cent glucose in saline, were injected to fasting patients within 4 to 5 minutes. Later, when small children and infants were included, 2 cc. of the hydrolysate per kilo, diluted with an equal volume of 5 per cent glucose in saline, were given. This represented about 12 mgm. amino acid N per kilo of body weight, enough to raise the plasma amino acid N level about 30 mgm. per 100 cc. if no amino acid were removed from the plasma. Blood samples were taken before and at 5 minutes, 15 minutes, 35 minutes, and 95 minutes after the completion of the injection. Urea N was determined on whole blood. The volume of packed red cells was measured and the plasma was analyzed for alpha amino acid N<sup>4</sup> and proteins. In addition, in some patients, the

urinary excretion of end-products of the metabolism of casein hydrolysate, given intravenously, was studied. When possible, urine was collected so that one or more control periods were obtained before the intravenous injection and several periods thereafter. Catheterization was avoided so urine collections were not always obtained at the desired intervals. The urine was analyzed for ammonia N, amino acid N, urea N, protein N, and total N, and in some instances, the pH was determined by glass electrode. Methods have been previously described (7). From these data, the urea and amino acid clearances could be calculated.

#### A. PLASMA AMINO ACID LOADING CURVES

##### *Results in normal children*

The children selected as controls, being convalescent hospital patients, cannot be considered ideally normal but clinically they were free from obvious liver or renal disease. Casein hydrolysate was injected intravenously and the behavior of the plasma amino acids and blood urea was observed. The plasma amino acid loading curves in 9 normal children are shown in Figure 2 and the complete pertinent data from one of them are presented in Table I. As examination of the

<sup>3</sup> Kindly furnished by Mead Johnson and Company.

<sup>4</sup> Throughout the paper, plasma amino acid N refers to alpha amino acid N as determined by the ninhydrin method.

chart shows, wide variation of fasting levels of plasma amino acid N in these normal children was observed. The plasma amino acid N level reached the initial value 35 minutes after the injection in one third of the group, and in all of the controls, the preinjection value was reached in 95 minutes. In 2 of the controls, the final value fell below the preinjection level. This unexpected phenomenon had also been observed in

normal dogs, Figure 1. Thus, no retention of amino acid was noted in any normal control 95 minutes after a load of intravenous amino acid. Blood urea levels remained essentially unchanged and shifts in hematocrit were slight, usually with a decrease immediately following the injection. The average plasma amino acid N level in normal fasting individuals has been reported to be 4.5 mgm. per 100 cc. In a series of 31 de-

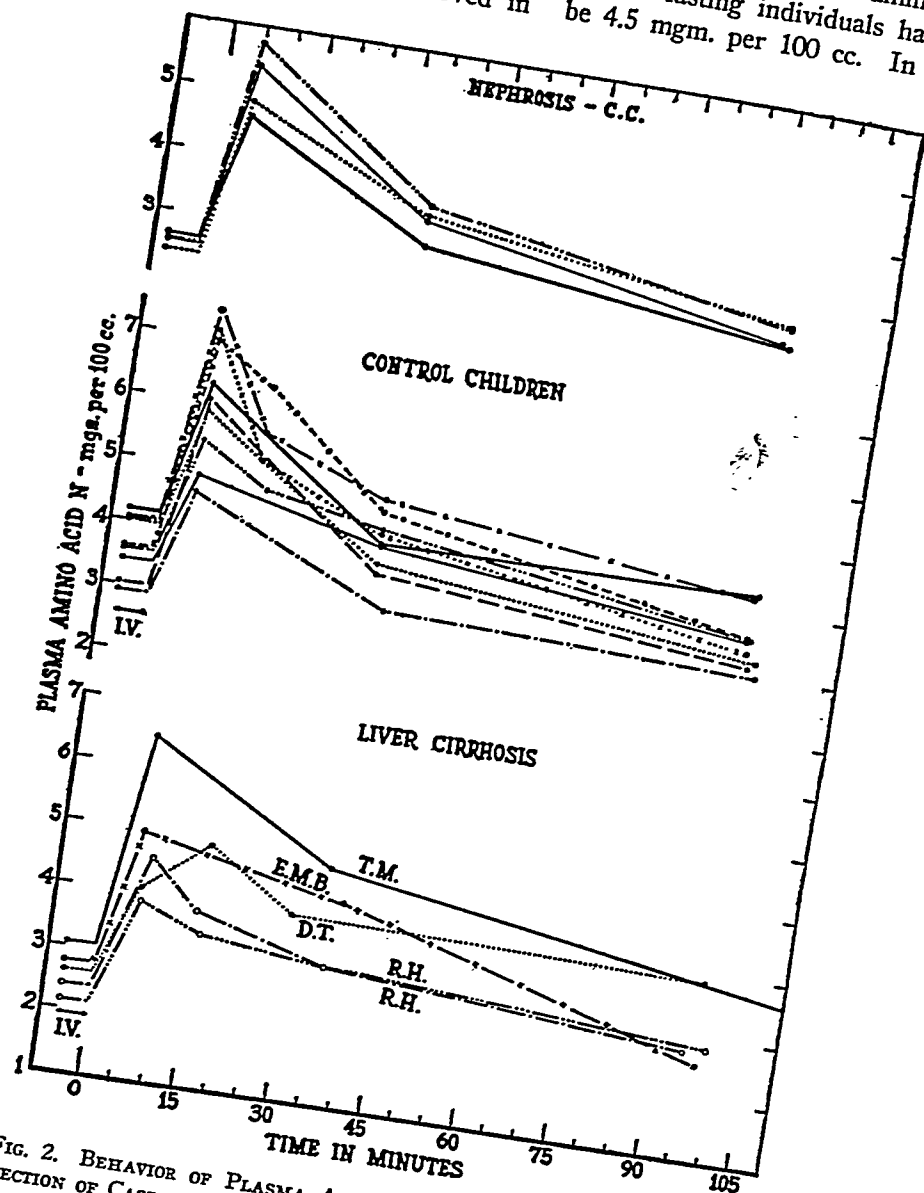


FIG. 2. BEHAVIOR OF PLASMA AMINO ACID N BEFORE AND AFTER THE INJECTION OF CASEIN HYDROLYSATE IN NORMAL CHILDREN AND IN PATIENTS WITH NEPHROSIS AND PORTAL CIRRHOSIS  
Retention becomes apparent if one places a guide line at the preinjection level for each curve.



TABLE I  
Analyses of blood before and after injections of casein hydrolysate in normal controls and patients with liver and kidney disease

Patient	Disease	Date	Plasma amino acid N				Blood urea N				Hematocrit		Serum albumin		
			Before i.v. Injection	After i.v. Injection			Before i.v. Injection	After i.v. Injection			Before	After 5 minutes			
				5 minutes	15 minutes	35 minutes		5 minutes	15 minutes	35 minutes				95 minutes	
			mgm. per 100 cc.				mgm. per 100 cc.				per cent		grams per 100 cc.		
A. S.	Control	March 4, 1941	3.57	5.39	4.76	4.42	3.58	13.86	12.78	14.24	12.69	14.53	38.7	36.9	4.51
R. F.	Nephrosis	February 6, 1940	2.87	4.30		3.22	2.61	10.94	10.20	9.28	10.48	9.63	35.1	34.5	1.69
		February 29, 1940	2.55	4.54		2.74	2.30	11.00	9.28	7.52	8.16	9.60	34.1	33.2	1.25
		March 7, 1940	2.91	4.46		3.24	2.64	8.35	7.52	8.31	9.06	32.7	32.5	1.74	
		March 15, 1940	2.80	4.82		3.24	2.85	9.28	9.67	9.36	9.60	32.2	32.2	1.74	
		October 28, 1940	2.84	4.26		3.45	2.95	8.42	8.12	8.68	8.05			1.36	
		March 25, 1941†	2.55	3.19	3.36	2.65	2.42	16.27	17.13	16.83	16.77	16.73	33.6	29.9	1.01
D. G.	Nephrosis†	April 3, 1941†	2.16	3.48	2.75	2.59	2.44	17.67	12.32	12.25	11.61	12.09	28.0	30.7	0.95
		May 15, 1941†	3.02	5.98	4.41	2.96	2.29						23.4	25.6	0.84
		March 4, 1940	2.86	9.54	4.71	4.46	3.28	23.37	26.31	26.78	28.47	26.26	33.6	34.2	1.03
M. H.	Chronic glomerulonephritis	January 21, 1941	3.75	5.89		3.92	2.89	26.10	26.13	27.33	28.65	38.4	36.2	4.33	
		October 2, 1941	3.66	5.90		4.04	3.65	27.41						4.23	
M. H.	Chronic glomerulonephritis	January 9, 1941	3.73	5.17	4.98		3.91*	40.85	40.39	39.99		40.90*	23.9	22.3	3.55
E. B.	Chronic glomerulonephritis	January 16, 1941	3.07	4.51	4.26	3.79	3.26	25.96	26.07	24.80	25.69	26.24	27.9	26.8	2.49
		January 7, 1942	3.47	7.69	5.62	4.87	3.92	36.80					26.3	24.8	2.63
R. S.	Nephrocalcinosis with chronic tetany	June 16, 1941	2.69	5.75	4.52	3.17	2.64	12.42	13.29	13.53	14.08		39.4	37.1	4.81
A. P.	Nephrocalcinosis with diabetes insipidus	April 29, 1941	3.24	6.29	4.97	4.20	3.24	11.82	12.01	13.33	13.86	11.74	42.9	41.3	4.27
M. K.	Renal glycosuria	July 30, 1940	3.38	5.44		4.06	3.56	14.79	15.08		13.65	12.72	42.3	39.5	4.85
D. T.	Portal cirrhosis	February 19, 1941	2.62	4.08	4.87	3.95	3.82	16.53	16.21	17.26	16.03	16.35	39.1	37.9	3.97
T. M.	Portal cirrhosis	July 15, 1941	3.07	6.47		4.76	3.61**	10.49					38.1		3.46
R. H.	Portal cirrhosis	April 1, 1941	2.41	4.56	3.80	3.19	2.72	8.84	8.75	9.04	9.45	9.55	38.6	40.6	2.50
		October 9, 1941	2.16	3.85	3.44	3.19	2.80	6.81				6.50	32.9	31.9	2.68
E. M. B.	Portal cirrhosis	April 10, 1942	2.79	4.96		4.27	2.56	12.90					36.3	34.5	4.94
T. S.	Glycogen storage disease	December 9, 1941	2.04	5.74	5.17	3.91	2.52					20.23	30.3	29.9	4.54
W. M.	Glycogen storage disease	January 14, 1942	4.55	7.16		4.61	2.92	11.08					21.7	21.4	4.27
F. B.	Hepatosplenomegaly	February 26, 1942	3.97	7.30	5.25	4.05	3.84	10.02	9.99	10.21	9.62	9.83	34.5	33.9	4.88
S. A.	Hepatomegaly	February 18, 1942	4.40	8.33	6.12	4.81	4.30	12.67		13.36	13.65	13.27	40.8		4.16
J. J.	Catarhal jaundice	February 11, 1941	3.37	4.94	4.02	3.25	3.72	10.78	11.79	11.18		10.39	38.2	36.5	4.70
F. T.	Diabetes with hepatomegaly without hepatomegaly	April 15, 1941 May 20, 1941	3.16 3.16	6.31 6.37	4.64 4.30	3.48 3.49	2.88 2.43	17.27	16.64	17.74	17.81	16.70 9.70	38.3	36.2	4.24

\* 60 minutes after i.v. injection.

\*\* 110 minutes after i.v. injection.

† Indicates nephrosis with poor renal function probably due to an associated glomerulonephritis.

terminations in 30 normal adults and children, Farr, McCarthy, and Francis (9) stated that the plasma amino acid N values ranged from 3.75 to 5.56 mgm. per 100 cc. with a standard deviation of  $\pm 0.46$ . In our hands, using the ninhydrin method of Van Slyke *et al.* (10), the values for 45 normal hospital children ranged from 2.92 to 4.63 mgm. per 100 cc. The mean was located at 3.60 mgm. per 100 cc.; the median at 3.53, with quartiles at 3.24 and 3.92 mgm. per 100 cc.

#### *Results in patients with liver disease and good renal function*

Some support was given to the assumption that retention of plasma amino acids indicates liver injury, by studies on 4 patients with portal cirrhosis and unimpaired renal function. The clinical diagnosis was verified by microscopic examination of liver tissue, obtained either by biopsy or at necropsy. The amino acid loading curves in 3 of these patients, included in Figure 2, showed a significant delay in clearing the blood of amino acids after the injection of casein hydrolysate. In the fourth patient, E. M. B., studies carried out early in the course of the disease demonstrated considerable retention in 35 minutes even though the preinjection level was reached in 95 minutes. Hence, in all of the patients with portal cirrhosis, plasma amino acid retention was noted.

In a group of 6 patients with hepatomegaly of other etiology than cirrhosis, plasma amino acid retention was shown in 2 instances, Table II. In one case of subsiding acute catarrhal jaundice, J. J., the retention was minimal and in one case of glycogen storage disease, T. S., there was moderate retention. To facilitate comparison, other

conventional liver function tests are presented in the table. For the sake of brevity, the data are interpreted in qualitative terms.

#### *Results in patients with nephrosis and good renal function*

Nine patients with nephrosis were studied in a similar fashion. With 4 of these patients, one or more curves were repeated at intervals as the opportunity presented itself. Figure 2 shows the amino acid loading curves on patient C. C. in the active stage of nephrosis. This patient had moderate edema, the serum albumin was 2 grams per 100 cc. and kidney function, as determined by the concentration test and by urea clearance, was normal. Four curves made during a period of 2 months were almost identical, and in all, the plasma amino acid level had returned to the original level within 95 minutes.

Another patient with nephrosis, R. F., had more extensive edema, a serum albumin of 1.6 grams per 100 cc., and a urea clearance of 113 per cent of the normal when the studies were begun. The complete data from this patient are included in Table I. He was under observation for one year, without remission of his disease, during which the clinical course was progressively downhill. The serum albumin fell to 1.1 grams per 100 cc., edema increased, and renal function suffered progressive impairment. The urea clearance at the end of the year had fallen to 45 per cent of normal. No enlargement of the liver was noted. During the year, 8 amino acid loading curves were made. All were within normal limits and at no time was retention of plasma amino acids found, in spite of the moderately severe

TABLE II

*Results of various liver function tests in patients with hepatomegaly. The quantitative tests are interpreted in a qualitative fashion and + to ++++ indicates deviation from the normal*

Diagnosis	Liver function tests									
	D. T.	R. H.	T. M.	E. M. B.	S. A.	F. B.	J. J.	F. T.	T. S.	W. M.
	Cirrhosis	Cirrhosis	Cirrhosis	Cirrhosis	Hepato- megaly	Hepato- spleno- megaly	Catarrhal jaundice	Diabetes; he- patomegaly	Glycogen storage disease	Glycogen storage disease
Bromsulfalein	++++	++++	+++	Normal	++++	++++	+	Normal	Normal	Normal
Galactose tolerance	++++	±	+++	Normal	++++	++	+	Normal	Normal	Normal
Cholesterol ratio	±	Normal	++++	Normal	Normal	+	+	Normal	Normal	Normal
Bilirubin clearance	+	±	±	±	++++	++++	+	Normal	Normal	Normal
Conjugation	Normal	±	±	±	+	+	+	Normal	Normal	Normal
Amino acid retention	++++	++++	+++	+	Normal	Normal	Normal	Normal	+	Normal
Serum albumin	3.97	2.68	3.46	4.58	4.16	4.83	4.70	4.24	4.50	4.27
Serum globulin	3.17	2.61	1.87	2.15	4.63	2.23	2.41	2.55	3.05	2.37
Serum bilirubin	0.78	0.60	0.82	0.52	5.82	5.56		1.01	0.50	

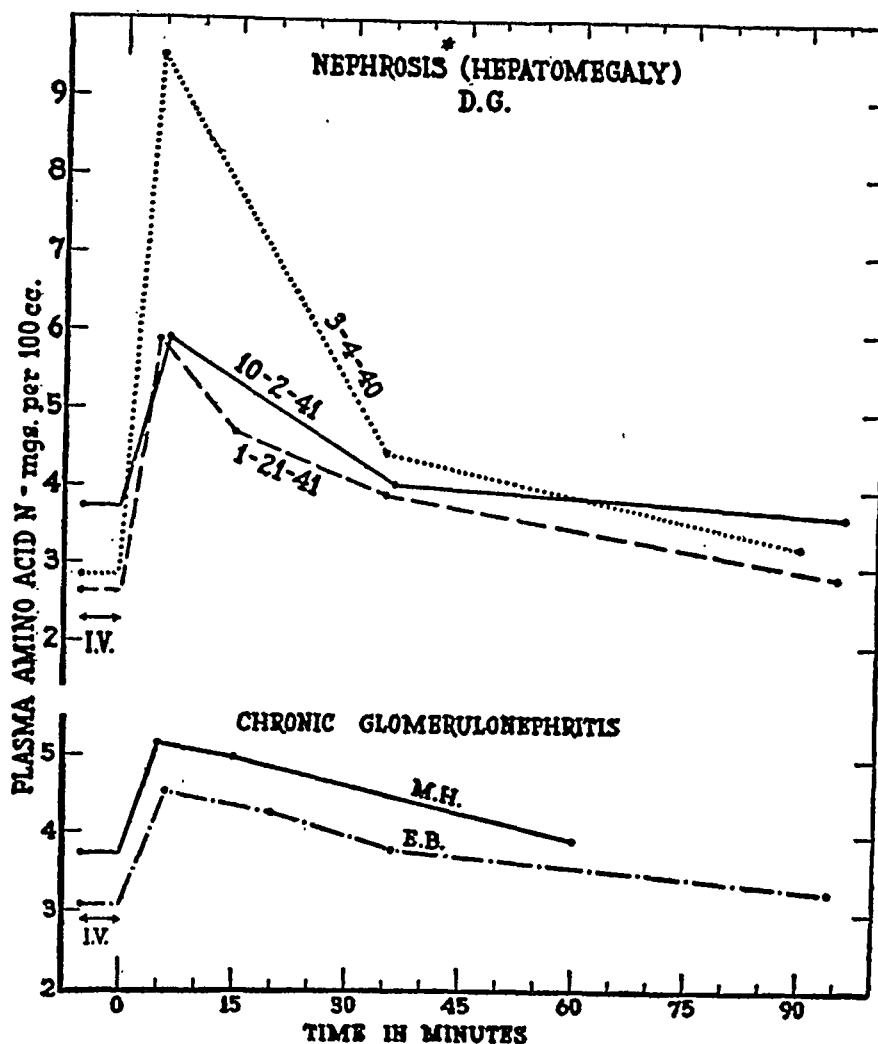


FIG. 3. BEHAVIOR OF PLASMA AMINO ACID N BEFORE AND AFTER THE INJECTION OF CASEIN HYDROLYSATE IN PATIENTS WITH IMPAIRED RENAL FUNCTION

impairment of kidney function. Here again it was possible to duplicate the curve repeatedly during a period of 12 months and the type of curve was similar to those found in the control group (Table I). Curves on 6 other nephrotic patients likewise showed no significant difference from those obtained in the controls. The only exception observed was in patient J. S. in whom slight plasma amino acid retention at 95 minutes was observed on one occasion and was not present later in the course of his disease. We can conclude from these somewhat limited studies that the nephrotic child handles an intravenous load of amino acids in approximately the same manner as a normal child.

#### *Results in patients with kidney disease and impaired renal function*

A few tests were carried out on 2 patients with chronic glomerulonephritis. Both children had

been under close observation for many years. Neither had ever developed a nephrotic component of the disease, *i.e.*, massive edema or low serum proteins with a reversal of the albumin/globulin ratio. In M. H., the urea clearance had fallen to 14 per cent, in E. B., to 35 per cent of the normal. No enlargement of the liver was evident on clinical examination in either patient. The amino acid loading curves shown in Figure 3 did not deviate significantly from the curves obtained in normal children, which suggests that severe kidney injury causes no delay in the clearance of amino acids from the blood stream after an intravenous load.

Patient D. G.<sup>5</sup> was first seen on January 13,

<sup>5</sup> Whether pure nephrosis exists or is a form of glomerulonephritis is an unsettled question and need not concern us here. To the writers, nephrosis is a distinct entity. A certain number of children with nephrosis have clinical glomerulonephritis superimposed, yet not

1939, with all the findings indicative of pure nephrosis. During the first year, he had numerous severe infections, namely, 6 episodes of bacteremia and 3 attacks of peritonitis, proven by bacterial cultures, from which he recovered with relatively short periods of chemotherapy. After 6 months of observation, hematuria appeared and nitrogen retention developed and persisted, the blood urea N being as high as 66 mgm. per 100 cc. It was thought that glomerular nephritis had been superimposed on the nephrosis. Marked hepatomegaly and splenomegaly developed. The etiology of the hepatomegaly was not clear but one may speculate that lipoid deposition, circulatory difficulties, and infection, all took part. The first loading curve was made on March 4, 1940, when hepatomegaly was present (Table I and Figure 3). The liver edge was palpated 5 cm. below the costal margin and the spleen, 4 cm. By this time, the blood urea N had fallen to 23 mgm. per 100 cc., and the urea clearance was 62 per cent of the normal. Serum albumin was recorded as 1.03 grams per 100 cc., and massive edema was present. As Figure 3 shows, the fasting plasma amino acid N was low in the nephrotic range. Following the injection, the plasma amino acid N rose sharply to 9.6 mgm. per 100 cc., a level much higher than had occurred in any control, and at 95 minutes, some retention persisted. Ten months later, on January 21, 1941, a second curve was made. Clinically, the child had had no severe infections since December 8, 1939, and had been free from edema since March 19, 1940. The liver edge could be palpated 2 cm. below the costal margin, the spleen, 1 cm., and kidney function had not changed. Serum albumin had risen to 4.42 grams per 100 cc. The fasting plasma amino acid N value was still low, but the rise following injection was less marked than that shown on the first curve and the final retention was about the same. Ten months after the second curve, October 2, 1941, the liver and spleen were barely palpable and physical examination indicated clinical recovery. However, the blood urea N remained slightly

elevated, at 27.4 mgm. per 100 cc., and the urea clearance was still only 61 per cent of normal. At this time, the fasting plasma amino acid N level was within normal limits and the loading curve was likewise normal. In this patient, the plasma amino acid N retention seemed to be correlated more closely with hepatomegaly and possible liver dysfunction than with renal impairment.

#### *Results in patients with nephrocalcinosis*

In 2 patients with nephrocalcinosis, without clinical evidence of hepatic disease, the amino acid loading curves were normal. In one of these patients, the urea clearance indicated moderate impairment, in the other, renal function was good (Table I).

#### B. URINARY EXCRETION STUDIES

In addition to the determination of the plasma amino acid N levels following the intravenous injection of casein hydrolysate, the urinary excretion of the end products of its metabolism was studied in some of the patients, to obtain, if possible, an insight into the rate of deamination. The data obtained enabled estimations to be made of the effect of injections of casein hydrolysate on renal urea and amino acid clearances as well. Pertinent results are presented in Table III.

Certain features were similar in all of the children examined, including the controls. No significant increase in urea clearance was observed following the injection. Only small amounts of amino acid were excreted so that the renal amino acid clearance remained minimal. The ratio of  $(\text{NH}_3\text{N} + \text{urea N})$  to total N.P.N. remained constant before and after the injection, so it was inferred that the casein hydrolysate was utilized as a metabolic protein.

#### *Results in normal children*

In 4 of the normal controls, urinary studies were carried out. In 3 of these children, during the period of observation following the injection of casein hydrolysate, the rate of ammonia and urea excretion was doubled. In one other control, during forced diuresis with a urine volume of 6 to 7 cc. per minute, the increase in ammonia and urea per hour was slight (11). In this patient, it is possible that the transient oliguria (a de-

all of these patients die in uremia. Some heal completely and remain healed, an outcome which is not compatible with a diagnosis of true chronic glomerulonephritis. A few die in uremia with anatomical findings characteristic of chronic glomerulonephritis.

TABLE III  
*Urinary nitrogen excretion following intravenous injection of casein hydrolysate in normal controls and patients with liver and kidney disease*

Patient	Disease	Date	Metabolism periods		Urine volume <i>cc. per minute</i>	Urine pH	Urinary excretion				Clearances		$\frac{\text{NH}_4\text{N} + \text{Urea N}}{\text{Total N.P.N.}}$		
				Total time			Urea N	Ammonia N	Amino acid N	Protein N	Total N	Urea		Amino acid	
A. S.	Control	March 4, 1941	Control No. 1	0 to 178	0.17	6.51	3.8	157.9	1.1	1.1	0	180.7	$\left. \begin{array}{l} 0.86 \\ 0.85 \end{array} \right\}$	53*	1.6
			Control No. 2	178 to 271	0.18	7.30	2.5	142.4	1.1	1.1	0	168.2		67*	2.1
			i.v. injection No. 3	271 to 348	0.36	6.66	6.5	252.5	2.7	2.7	0	300.7		61*	2.1
			i.v. injection No. 4	348 to 407	0.18	6.03	6.5	162.0	1.6	1.6	0	187.8			
E. Be.	Control with diuresis	February 3, 1942	Control No. 1	0 to 59	6.73	7.33	12.0	296.8	0.2	0.2	0	355.4	$\left. \begin{array}{l} 0.83 \\ 0.86 \end{array} \right\}$	72	0.3
			i.v. injection No. 2	59 to 148	0.89	6.45	15.8	251.3	7.6	7.6	0	324.8		49*	1.4
			Control No. 3	148 to 198	6.90	6.85	14.9	310.6	1.8	1.8	0	330.6		77	1.2
			i.v. injection No. 4	198 to 239	7.63	6.63	14.8	310.9	1.8	1.8	0	329.5		77	1.2
R. F.	Nephrosis	October 28, 1940	Control No. 1	0 to 215	0.47		4.8	88.0	1.2	1.2	30.0	132.7	$\left. \begin{array}{l} 0.83 \\ 0.82 \end{array} \right\}$	45*	2.1
			Control No. 2	215 to 370	0.68		7.6	157.4	2.5	2.5	69.1	265.2		40*	1.2
			i.v. injection No. 3	370 to 495	1.10		11.5	178.1	2.2	2.2	75.2	296.9		42*	1.2
			i.v. injection No. 4	495 to 625	1.04		8.9	173.0	1.8	1.8	73.9	282.9			
N. T.	Nephrosis	April 3, 1941†	Control No. 1	0 to 175	1.04	6.93	3.9	147.6	2.2	2.2	127.6	290.1	$\left. \begin{array}{l} 0.90 \\ 0.85 \end{array} \right\}$	16*	2.0
			Control No. 2	175 to 301	1.30	7.23	3.4	168.5	2.5	2.5	141.9	331.0		16*	3.8
			i.v. injection No. 3	301 to 363	0.85	7.23	2.7	128.8	2.1	2.1	110.3	264.2		27	
			i.v. injection No. 4	363 to 413	1.60	7.00	3.5	135.6	2.9	2.9	116.7	275.0			
N. T.	Nephrosis	September 29, 1941	Control No. 1	0 to 85	1.14		4.9	212.2	2.3	2.3	16.9	263.3	$\left. \begin{array}{l} 0.87 \\ 0.86 \end{array} \right\}$	49*	2.9
			Control No. 2	85 to 160	1.48		6.2	293.1	3.1	3.1	17.0	352.0		88	4.7
			i.v. injection No. 3	160 to 250	1.94		16.9	261.2	5.2	5.2	12.1	309.3		73	
			i.v. injection No. 4	250 to 310	4.30		17.5	305.0	6.4	6.4	11.9	379.0		83	
D. G.	Nephrosist	January 21, 1941	Control No. 1	0 to 177	0.24		6.4	221.5	0.9	0.9	0	239.3	$\left. \begin{array}{l} 0.93 \\ 0.88 \end{array} \right\}$	48*	1.9
			Control No. 2	177 to 294	0.29		3.9	142.8	0.6	0.6	0	153.5		28	1.2
			i.v. injection No. 3	294 to 428	1.59		7.2	183.3	1.2	1.2	0	212.7		43	
			i.v. injection No. 4	428 to 458	2.93		9.4	270.6	0.8	0.8	0	290.4		47	
D. G.	Nephrosist	October 2, 1941	Control No. 1	0 to 210	0.95		9.4	274.2	0.9	0.9	0	303.7	$\left. \begin{array}{l} 0.93 \\ 0.88 \end{array} \right\}$	47	1.9
			i.v. injection No. 2	210 to 280	0.41		11.1	291.8	0.6	0.6	0	305.1		45	1.2
			i.v. injection No. 3	280 to 360	2.05		39.2		1.1	1.1	0	340.5			

TABLE III—Continued

Patient	Disease	Date	Metabolism periods			Urine volume cc. per minute	Urine pH	Urinary excretion					Clearances		NH <sub>4</sub> N+Urea N Total N.P.N.
			Total time	minutes	Urea N			Ammonia N	Amino acid N	Pro- tein N	Total N	Urea	Amino acid		
E. B.	Chronic glomerulo- nephritis	January 16, 1941	Control	No. 1	0 to 135	1.24		357.3	5.6	2.5	50.4	463.5			0.87 0.84
			Control	No. 2	135 to 295	0.58		197.0	2.1	1.5	37.6	265.1	19*	1.3	
			i.v. injection	No. 3	295 to 445	0.71		218.0	4.0	2.2	34.8	294.8	19*	1.5	
			i.v. injection	No. 4	445 to 495	0.58		179.5	3.0	2.1	28.4	244.8	18*		
M. H.	Chronic glomerulo- nephritis	January 9, 1941	Control	No. 1	0 to 180	1.29		209.8	3.3	4.6	68.7	314.0			0.87 0.87
			Control	No. 2	180 to 240	0.90		151.7	2.4	2.9	61.7	225.0	7*	1.6	
			i.v. injection	No. 3	240 to 315	0.76		127.8	1.8	3.4	57.0	202.4	7*	1.8	
			i.v. injection	No. 4	315 to 355	1.10		158.6	3.3	3.9	61.2	247.5	7*		
D. T.	Portal cirrhosis	February 19, 1941	Control	No. 1	0 to 139	0.38	5.55	293.3	19.6	2.5	0	347.1	61*	3.3	0.85 0.88
			i.v. injection	No. 2	139 to 274	0.42	5.84	393.5	23.2	3.9	0	447.5	75*		
R. H.	Portal cirrhosis	April 1, 1941	Control	No. 1	0 to 241	0.13	5.51	112.7	9.1	1.6	0	136.0	57*	3.6	0.83 0.78
			i.v. injection	No. 3	241 to 512	0.11	5.59	77.5	4.7	1.3	0	92.3	63*		
S. A.	Hepatomegaly	February 18, 1942	Control	No. 1	0 to 241	0.24		188.2	17.0	2.0	0	242.5			0.79 0.77
			Control	No. 2	241 to 397	0.22		174.8	14.8	1.9	0	216.9	65*	2.1	
			i.v. injection	No. 3	397 to 539	0.25		188.5	11.8	2.8	0	247.6	66*	1.2	
			i.v. injection	No. 4	539 to 619	0.56		263.0	7.0	1.7	0	335.3	60*		
F. B.	Hepatosplenomegaly	February 26, 1942	Control	No. 1	0 to 243	0.15		135.7	15.8	1.5	0	161.1			0.84 0.83
			Control	No. 2	243 to 363	0.15		141.5	14.3	1.7	0	170.3	84*	2.6	
			i.v. injection	No. 3	363 to 528	1.37		232.8	16.8	3.9	0	281.0	78	2.5	
			i.v. injection	No. 4	528 to 585	1.08		206.9	7.8	2.9	0	245.5	70		
F. T.	Diabetes; hepatomegaly	April 15, 1941	Control	No. 1	0 to 240	0.33	5.00	235.9	15.0	1.4	0	266.5			0.89 0.83
			Control	No. 2	240 to 420	0.94	4.97	259.8	14.3	1.2	0	290.6	38*	1.0	
			i.v. injection	No. 3	420 to 543	1.12	4.71	283.1	20.6	5.7	0	344.4	57	2.8	
			i.v. injection	No. 4	543 to 620	0.62	4.78	213.9	17.9	2.8	0	252.5	39*		
J. J.	Catarrhal jaundice	February 11, 1941	Control	No. 1	0 to 180	0.27	5.46	146.0	18.4	1.6	0	184.0	55*	1.5	0.80 0.81
			Control	No. 2	180 to 300	0.43	5.57	183.1	17.3	4.2	0	228.5	54*	1.5	
			i.v. injection	No. 3	300 to 365	0.28	5.86	187.6	14.9	1.9	0	239.1	66*	1.5	
			i.v. injection	No. 4	365 to 435	0.51	5.80	238.5	18.9	1.9	0	287.1	64*		

\* Calculated as standard clearances. Unmarked calculated as maximum clearances.

† Indicates nephrosis with poor renal function probably due to an associated glomerulonephritis.

crease in volume from 6 to 7 cc. to 0.9 cc. per minute) which resulted from the injection of casein hydrolysate masked the findings.

#### *Results in patients with kidney disease*

Four patients with nephrosis in whom kidney function was normal were studied. As in the normal controls, the rate of ammonia excretion was increased two- or threefold but, in contrast to the controls, only a slight increase in the rate of urea excretion was observed.

The manner in which one patient with nephrosis, R. F., metabolized the casein hydrolysate was studied repeatedly. The data from 2 of the studies appear in Table III. As previously described, urinary studies carried out on October 28, 1940, when renal function was good, demonstrated a twofold increase in the rate of ammonia excretion and a slight increase in urea excretion per unit of time. On April 3, 1941, the study was repeated. The clinical course was progressively downhill and at this time, the urea clearance had decreased to 31 per cent of the normal. Following the injection of casein hydrolysate, there was no increase in the amount of ammonia excreted in unit time, while the rate of urea excretion actually diminished. These results were confirmed on 2 subsequent occasions. Thus, this patient when first studied was able to respond to the injection of casein hydrolysate with an increased rate in the excretion of ammonia. Later, as might be expected with diminished kidney function, this ability was lost.

In 2 patients, E. B. and M. H., with chronic glomerulonephritis and severely impaired renal function, with clearances of 35 per cent and 14 per cent of the normal, respectively, as might be predicted, no increase in the rate of ammonia or urea excretion was noted following the injection of casein hydrolysate.

#### *Results in patients with liver disease and good renal function*

Two patients with portal cirrhosis and plasma amino acid retention were available for urinary studies. Before hypoproteinemia had developed, patient D. T. showed a moderate increase in the rate of urea and ammonia excretion after the injection of casein hydrolysate. In patient R. H., with severe hypoproteinemia in the later stages

of cirrhosis, only a slight increase in the rate of urea and ammonia excretion was observed, which suggested a reduction of deamination.

In patient, J. J., with catarrhal jaundice, in whom moderate plasma amino acid retention had been observed, there was no increase in the rate of ammonia excretion and a considerable lag in the rate of urea excretion. In 2 jaundiced patients, S. A. and F. B., with hepatomegaly of unknown origin, in whom no plasma retention had occurred, a lag in the rate of urea excretion was noted in one but not in the other. In neither patient was there an increase in the rate of ammonia excretion. Thus, 3 jaundiced patients with greatly enlarged livers and no disease of the kidney failed to show any increase in the rate of ammonia excretion above the catabolic level. The data from 2 of these patients suggested a reduction in the rate of deamination; but in only one, J. J., was plasma amino acid retention found.

In patient F. T., with marked hepatomegaly associated with poorly regulated diabetes, in whom the conventional liver function tests were normal, the rates of ammonia and urea excretion were moderately increased.

#### DISCUSSION

A common denominator between the experimental dog and the nephrotic patient is hypoproteinemia, even though the etiology of the hypalbuminemia is not the same. The nephrotic child remains in positive nitrogen balance when fed protein but is unable to replenish his depleted serum proteins (12), while the experimental dog, with intact ability to generate serum proteins, fails to do so because protein is withheld from the diet. It is commonly assumed that the liver is an essential organ for the synthesis of serum proteins. Since the nephrotic patient can maintain neither adequate serum proteins nor normal plasma amino acid N levels, it seemed logical to study the metabolism of amino acids in these patients with a view to uncovering possible liver dysfunction.

The manner in which normal children handle an intravenous load of casein hydrolysate was determined. Most of the injected amino acid was removed from the blood stream immediately by diffusion into the tissues, the plasma amino acid N rose to twice the normal level 5 minutes after

completion of the injection, and within 95 minutes, the preinjection level was regained in all patients. Loading curves in the nephrotic patients were similar to those obtained in the normal controls.

In contrast, loading curves in 4 patients with portal cirrhosis showed plasma amino acid retention. In 2 patients, hypoalbuminemia was present. In 3, most of the conventional liver function tests indicated severe impairment; in one patient with minimal retention, seen early in the course of the disease, most of the conventional tests were still normal. All of the patients had normal kidney function. In other words, no plasma amino acid retention was found in the normal dog, the normal child, or the hypoproteinemic child with pure nephrosis; while retention was observed in the hypoproteinemic dog with liver injury and in the patients with portal cirrhosis.

In the children with kidney disease and no clinical evidence of liver disease, the loading curves showed no plasma amino acid retention. Hence, it seemed that kidney impairment, even of severe degree, did not interfere with the removal of plasma amino acid when a load was placed on the mechanism. Of passing interest was the fact that the patient with renal glycosuria did not excrete significantly larger amounts of amino acid than the normal controls or other patients.

In the group of patients with hepatomegaly, retention of plasma amino acid following an injection of casein hydrolysate could not be correlated with other tests of liver function to the same degree as in the patients with liver cirrhosis, nor did the additional test of ability to dispose of a load of intravenous amino acids offer a solution to those in search of an infallible test of liver impairment. Actually, the numerous functions of the liver may be selectively impaired as are the functions of the kidney.

In one patient with hepatosplenomegaly and kidney impairment, moderate plasma amino acid retention was found when the liver and spleen were enlarged and disappeared as recovery ensued, even though some impairment of kidney function persisted. In contrast, in chronic glomerulonephritis with severely impaired renal function without liver involvement, no tendency to retention was observed.

In the hypoproteinemic dog, studies of urinary excretion of ammonia, urea, and amino acid, fol-

lowing an intravenous injection of casein hydrolysate under standard conditions, suggested that the plasma amino acid retention was probably due largely to a reduction in the rate of deamination.

In the normal child, following intravenous injection of 12 mgm. amino acid N per kilo, the forces of diffusion into the tissues and deamination were such that the preinjection plasma amino acid N level was reached between 35 and 95 minutes. Most of the amino acid diffused rapidly into all of the tissues and subsequently reached the liver where it was rapidly deaminized to form ammonia, which in turn was synthesized to urea.<sup>6</sup> At normal urine flows in 3 control children, the rates of ammonia and urea excretion per hour were doubled within the period of observation following the injection. Loss of amino acid through the kidney during the injection was minimal. During the period of observation, blood urea levels did not vary significantly, which indicated that the equilibratory mechanism for maintenance of normal blood urea values was not strained by the amount of amino acid given. The observed increase in the rates of ammonia and urea excretion, above the level of *catabolism* in the absence of acidosis, was considered to represent the balanced response of a normal liver and normal kidneys, in a child, to the injection of 12 mgm. of amino acid N per kilo.

Interpretation of the metabolism studies in the patients was more difficult than in the experimental dog. In the normal dog, the substantial increase in the rate of urea excretion above the catabolic level, immediately following an injection of amino acid, was considered an indication of the speed of deamination. As hypoproteinemia was induced, the decrease in the rate of urea excretion was thought to represent (a) immediate utilization of amino acids to replenish depleted tissues, and (b) a decreased rate of deamination of the remaining fraction which in spite of (a) was sufficient to produce plasma amino acid retention. In the experimental dog, presumably no serious interference with kidney function had occurred,

<sup>6</sup> At the present time, it is commonly accepted that practically all deamination occurs in the liver unless base depletion creates an unusual demand for ammonia formation by the kidney (13 to 15). If renal deamination occurs, the chances on the basis of organ size indicate that it will prove to be of relatively small quantitative importance (1).



since the urea clearance did not vary significantly throughout the period of deproteinization and was only slightly lower than when the dog was fed protein (7); the urine remained normal to microscopic examination throughout the course; and no evidence of morphological injury to the kidney was found post mortem. Obviously, in patients with impaired renal function, the rate of urea excretion could not be used to estimate the speed of deamination. Hence, evaluation of interference with deamination could be attempted only in those patients with good renal function. In the series reported here, this was limited to patients with pure nephrosis and liver disease.

In 4 patients with nephrosis and no plasma amino acid retention, in whom urinary studies were made, the rate of urea excretion was slightly increased, but much less so than in the normal controls. This may be interpreted as a slight reduction in deamination, but it is clear that if reduction in deamination occurred, it was not sufficient to result in plasma amino acid retention.

In only one of 2 patients with portal cirrhosis, in whom urinary studies were made, did the failure to excrete urea above the catabolic level suggest a reduction in deamination. In 2 out of 3 jaundiced patients, there was a lag in the urea excretion suggesting delay in deamination but in only one of them was plasma amino acid retention observed. Although urinary studies in patients with hepatomegaly in whom plasma amino acid retention was observed were too limited in number to permit a definite conclusion, the data obtained are compatible with the suggestion that deamination is delayed.

Nash and Benedict in 1921 (16) proved by a series of ingenious experiments that urinary ammonia is formed only in the kidney. Bliss (17) demonstrated that certain amino acids can serve as the precursors for ammonia formation in the kidney of acidotic dogs. The subject of ammonia formation and transport in the blood stream is still controversial and has been reviewed recently (1, 18, 19). The data obtained in normal children and in patients with kidney disease are compatible with the theory of ammonia formation proposed by Nash and Benedict. In contrast, the patients with liver disease and unimpaired kidney function, as judged by urea clearance, failed to excrete ammonia at the expected rate, even though

an acidosis was present. Present knowledge offers no satisfactory explanation of the observed fact.

#### SUMMARY

In 45 normal hospital children, the fasting plasma amino acid N ranged from 2.92 to 4.63 mgm. per 100 cc.

Intravenous injections of casein hydrolysate were given to normal children, and to patients with nephrosis, nephritis, and liver disease. In 4 children with portal cirrhosis, a delay in clearing the plasma of amino acids was observed following the injection. In 2 of 7 patients with liver disease other than cirrhosis, a moderate delay was noted. In one patient with hepatosplenomegaly and impaired renal function, retention of plasma amino acid was noted, which disappeared when the enlargement of liver and spleen abated.

On the other hand, 8 of 9 patients with nephrosis, 2 patients with chronic glomerular nephritis, and 3 patients with various other types of kidney disease cleared the plasma of amino acid as quickly as the normal controls. Severe impairment of renal function offered no obstacle to the rapid clearing of injected amino acid from the plasma, while delay in plasma clearance could be related to liver dysfunction.

Urinary excretion studies were carried out in some of the patients following the injection. Representative patients of each of the above groups showed no significant increase in urea clearance. Only small amounts of amino acid were excreted so that the amino acid clearance was low, a few cc. per minute per sq. M.

The rate of urea excretion in nephrotic and cirrhotic patients and in those with other forms of liver disease, although increased slightly above the catabolic level, was not so great as that observed in the controls. This might indicate a reduction in deamination.

The rate of ammonia excretion in normal controls and in patients with nephrosis was doubled immediately after the injection. In 5 patients with severe impairment of renal function, no increase in the rate of ammonia excretion was noted. Although 2 patients with cirrhosis showed a slight increase in ammonia excretion, 3 jaundiced patients with marked hepatomegaly and normal renal function failed to excrete ammonia above the catabolic level.

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# PLASMA CLOT TENSILE STRENGTH MEASUREMENT: ITS RELATION TO PLASMA FIBRINOGEN<sup>1</sup>

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In the course of experimental work on the problem of autologous plasma clot suture of nerves (1), the desirability of *in vitro* tests of plasma clots became apparent and an instrument and method which might serve the purpose were devised. Since the procedure seemed likely to be useful to other workers, it was reported (2) while we were still accumulating the data now presented.

Methods for measuring the tensile strength of clots have been used previously. Fonio (3) centrifuged 6 cc. quantities of blood mixed with 2 cc. of 0.75 per cent  $MgSO_4$  to retard clotting, allowed spontaneous clotting of the supernatant plasma, then cut the plasma clot free of the cellular portion below. After compressing the clot in a special press to a  $0.5 \times 1.0$  cm. disc, it was fixed in ether and then clipped above and below in its shorter dimension. Weights were then added until the clot broke and the maximal weight held recorded. The tensile strength of such fibrin discs was found to vary in individuals, the normal range being from about 250 to 350 grams. In diseased individuals, he found that the values obtained were often greater or less than the normal (4). He did not attempt to correlate this variation with any property or constituent of the blood. Kristenson (5) used a slightly different technique; he did not add any anticoagulant and allowed the clot to retain its natural shape. Clots were formed from chilled plasma, in test-tubes of uniform bore, and fixed in ether. He suggested that variations in clot tensile strength are dependent on varying amounts of fibrinogen present as well as on other factors.

In the present paper, some factors affecting the tensile strength of plasma clots have been inves-

tigated, using a technique somewhat modified from that described by Kristenson. An attempt has been made to avoid objections such as can be raised against the work of Fonio and of Kristenson, by omitting the use of any fixative, by testing the clots in triplicate or quadruplicate, and by examining a larger number of specimens in order to attain at least minimal statistical validity.

## METHOD

The method used by us consists essentially of allowing coagulation of 1 cc. of plasma to take place in test-tubes of uniform bore (0.75 cm. in diameter), at  $37.5^\circ C.$ , in a water bath. No anticoagulant was added (unmodified plasma), premature clotting being prevented by drawing the blood into a chilled, oiled syringe and immediately transferring it to paraffin-lined tubes, packed in ice, for centrifugation to separate the plasma.

The clots were removed from the test-tubes by carefully rimming them with a cool platinum wire and then either enmeshing the tip of the wire in the uppermost portion or heating the tip of the wire so that the clot would adhere to it and could thus be lifted out.

The apparatus used (2) is essentially a rigid frame to which is attached a rubber-sleeved hemostat which clips the upper end of the clot and suspends it in front of a millimeter rule. A light rubber-sleeved clip grasps the lower end of the clot. To the lower clip are appended 5 or 10 gram weights, one at a time, until rupture of the clot occurs. The increase in length is measured as each weight is added.

The tensile strength is defined in this paper as the maximum weight held in grams (including the weight of the clip) before the final increment caused rupture of the clot.

## PRECAUTIONS

Variability in readings obtained in testing clots, formed from the same specimen of plasma under the same conditions, is to be expected on the basis of experimental error intrinsic to the method.

Care must be taken to avoid damage to the clot when removing it from the tube or applying the clip. One's suspicion should be aroused if in the test, when the weight is applied, the rupture does not occur near the mid-point where "necking" ordinarily is maximal. When the clot is clipped, its entire width must be included because the tensile strength varies with the cross-sectional area. On the other hand, since our results show that the

<sup>1</sup> This study was aided by a grant from the Committee on Scientific Research of the American Medical Association.

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length of the clot has very little effect on the tensile strength reading, should the clot be torn when the clip is applied, there is no objection to reapplying it if enough clot remains for convenient testing. Also, in order to avoid shearing, the clot must be clipped exactly transversely, and some practice is required before one learns to apply the weights without tugging. In general, improper technique as outlined above tends to give a false low value for the tensile strength.

In most of our determinations, an interval of 15 minutes was allowed to elapse between clotting and the first test. This was done because it was not infrequently observed (especially in experiments where anticoagulant substances had been added to plasma) that the clot did not appear to have achieved what was considered its maximum stability until approximately such a period had elapsed. Thus, after clotting had progressed sufficiently at the surface to prevent deformation on tipping ("tip test"),

the base of the clot might still show merely strand-like clotting and a more transparent or "fluid" appearance. When such clots were removed from the tubes, their lower parts were found to consist merely of fibrin sacs, filled with unclotted plasma. Accordingly, we deemed it advisable to make a quantitative study of the tensile strength of the clots in relation to their age. Ten series of clots were tested, in duplicate and triplicate, at the following ages: 0, 3, 6, 9, 12, 15, 20, and 30 minutes, 0 representing the time when the tip test first became positive. (Older clots were not tested as it had previously been found that there was no variation among clots whose ages ranged from  $\frac{1}{2}$  hour to 24 hours.) Since the clots in these experiments were prepared from plasmas from different individuals, they varied considerably in tensile strength. In order not to give undue weight to those clots with high tensile strength, when computing the average tensile strength of any given specimen at any

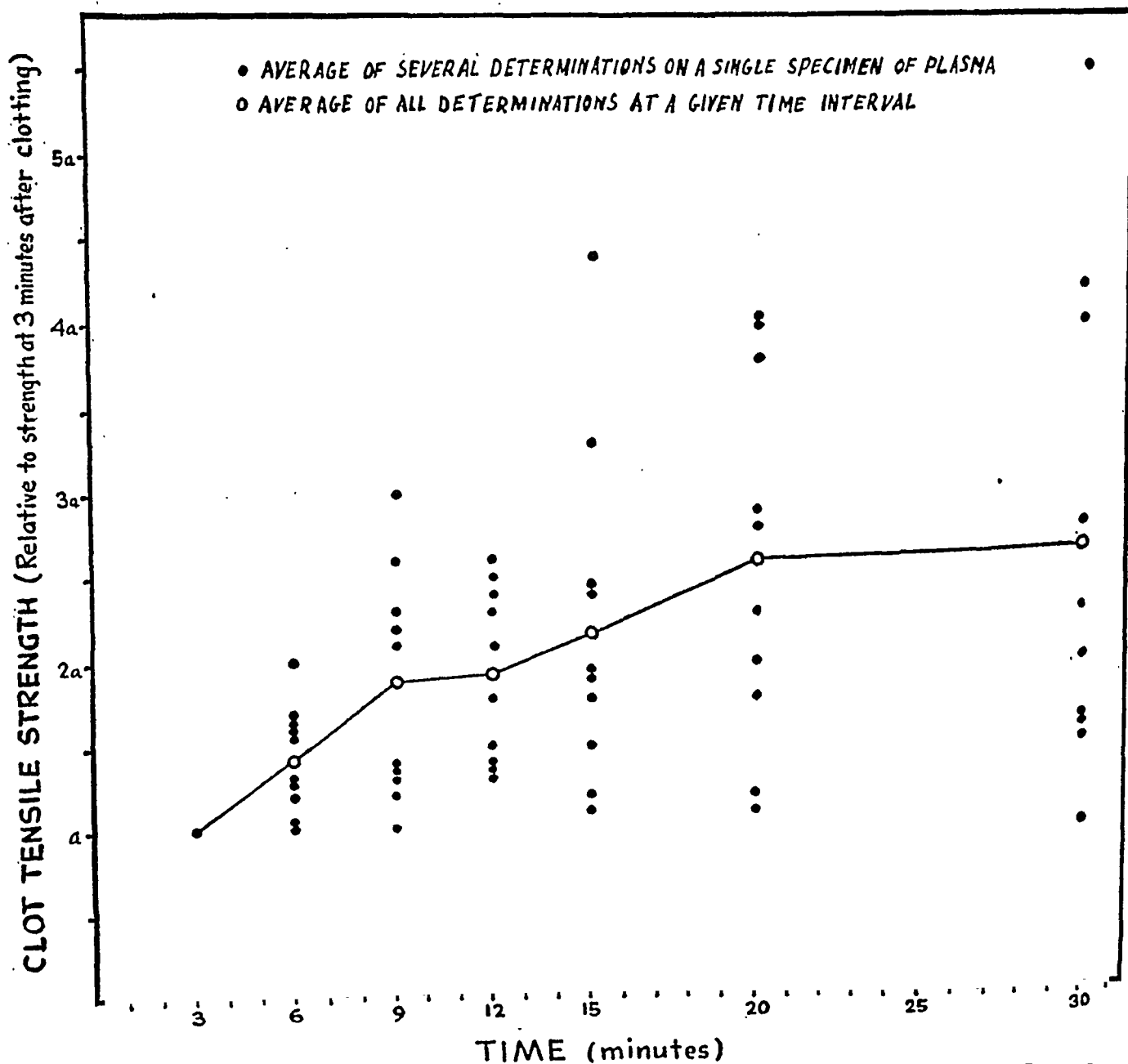


FIG. 1. RELATION OF CLOT TENSILE STRENGTH TO THE INTERVAL BETWEEN CLOTING AND TESTING

TABLE I  
Plasma clot tensile strength (in grams) in 11 normal individuals

Subjects and sex		ts and cr†	Time of test in minutes														Average
			5 to 9	10 to 14	15 to 19	20 to 29	30 to 39	40 to 49	50 to 59	60 to 69	70 to 79	80 to 89	90 to 99	100 to 109	110 to 119	120 to 129	
1	Y.M. M.	ts															
2	B. M.	ts			65			35	45	45							41.6
3	P.C. M.	ts					75										
4	T.L.X. M.	ts cr				65	55	55									65.0
5a*	A. E. M.	ts cr	15 +				30 +										58.3
b*		ts cr					40 0									20 +	28.3
6a†	V.K. M.	ts cr		70 0			40 +			65 +++	35 0						38.7
b†		ts cr			35 0						50 0						53.3
		ts cr						55 +++									
		ts cr						45 +++		60 +++	50 +++						50.0
c†		ts cr						35 ++++		35		35					37.5
7	T.L.M. M.	ts cr				45 0	30 0	40 +++	35 +++								37.5
8	R.E. F.	ts cr		55 ++	35 0			45 +++									45.0
		ts cr	30 ++		30 ++	25 0	20 ++	45 ++++	30 ++++								31.2
		ts cr							15 0								
9	A.G. M.	ts cr							55 0								46.3
10	H. M.	ts cr			40 0	50 0	50 0		45 +								45.0
11	B.E. F.	ts cr			45 0		35 ++				55 ++++						60.0
		ts cr		70 +++	35 ++	45 ++++	145 ++++	45 ++++	45 0								
		ts cr			35 +++												

† ts indicates tensile strength and cr, clot retraction (+ slight, ++ moderate, +++ severe, marked).

\* Repeat tests on same subject at interval of several days.

given time, this

† ts indicates tensile strength and cr, clot retraction (+ slight, ++ moderate, +++ considerable, and ++++ marked).  
\* Repeat tests on same subject at interval of several days.

† Repeat tests at weekly intervals.

## RESULTS

### I. Normal variations in clot tensile strength

given time, this was expressed in relation to its strength at 3 minutes taken as a base (a). Figure 1 shows the relative increment in clot tensile strength with the age of the clot. It can be seen that there is an increase in strength until the age of the clot reaches 20 minutes, after which there is hardly any change.

Table I gives the data obtained from the study of 11 normal human subjects (of ages 6 to 53

years). Specimens 1, 4, and 7 were from healthy young blood donors; 2, 3, and 10 were from healthy males who had undergone minor surgical procedures on the hospital ward; 5, 6, and 9 were from laboratory workers; 8 and 11 were from females studied in the Out-Patient Department and reported as free of organic disease.

The table demonstrates that in normal individuals, by this method, there is variation of clot

tensile strength, but within a narrow range. The mean clot tensile strength of normals in this group is 45.5 grams. There was no apparent correlation between clot tensile strength and age or sex. This lack of correlation was substantiated by all data obtained subsequently. In the table, we have also recorded the degree of retraction of each clot tested and it can be seen that this varied considerably. There appears to be no relation between

TABLE II

*Plasma fibrinogen in grams per 100 cc. plasma and clot tensile strength in grams held*

Patient	Sex	Diagnosis	Fibrinogen*		Tensile strength and clot retraction, (quadruplicate determinations)†					Average tensile strength
L. H.	M	Involutional melancholia	0.27	0.40	55 ..... +++	55 ..... 0	65 ..... ++	55 ..... +++		57.5
M. B.	M	Arteriosclerosis	0.29		55 ..... ++	55 ..... 0	65 ..... +++			58.3
R. S.	M	Inguinal hernia	0.31	0.37	45 ..... ++	35 ..... ++	50 ..... +++	55 ..... +		46.2
M. A.	M	Inguinal hernia	0.32		55 ..... 0	75 ..... 0	75 ..... +	35 ..... 0	65 ..... 0	61.0
E. B.	M	1. Possible typhoid fever 2. Possible trichinosis	0.40		45 ..... +	55 ..... ++	55 ..... ++	55 ..... 0		52.5
R. W.	F	Pulmonary tuberculosis, healed	0.40	0.44	65 ..... ++	55 ..... +++	75 ..... +++	75 ..... +++		67.5
J. P.	M	Myelogenous leukemia	0.42		95 ..... 0	45 ..... 0	95 ..... 0	95 ..... ++		80.0
M. A.	F	Pregnancy	0.43	0.48	115 ..... +++	65 ..... +++	145 ..... +++	100 ..... 0		106.2
A. W.	M	Nephritis	0.44	0.48	55 ..... +++	75 ..... +++	85 ..... +++	35 ..... +++		62.5
F. N.	M	Acute rheumatic fever	0.46	0.63	55 ..... ++	85 ..... +++	65 ..... ++	65 ..... 0		67.5
K.	F	Pregnancy	0.50	0.54	135 ..... +++	95 ..... 0	105 ..... 0	95 ..... 0		107.5
C. O.	F	Pulmonary tuberculosis	0.505	0.65	145 ..... +++	75 ..... +++	135 ..... +++	75 ..... +++		107.5
M. A.	M	Fracture of left femur	0.525		85 ..... +++	85 ..... +++	75 ..... +++			81.6

TABLE II—Continued

Patient	Sex	Diagnosis	Fibrinogen*		Tensile strength and clot retraction (quadruplicate determinations)†					Average tensile strength
L. L.	M	Adenocarcinoma, hepatic flexure	0.55		105 +++	85 ++++	105 +++	95 +++		97.5
S. S.	M	Acute rheumatic fever	0.56		175 +++	95 +++	155 +++	95 +++		130.0
A. A.	M	Inguinal hernia	0.57		75 ++	65 +++	75 +++	65 +++		70.0
M. C.	M	Carcinomatosis	0.585		165 +++	85 0	155 +++	145 +++		137.5
P. C.	M	Carcinoma of rectum	0.595	0.79	145 +++	65 0	85 ++++	105 +++	95	99.0
C. B.	F	Retroperitoneal malignancy	0.64		155 ++	85 +++	95 +++	105 ++		110.0
K. B.	M	Pulmonary carcinoma	0.675		155 +++	135 +++	85 +++	95 +++		117.5
K. D.	M	Cervical adenitis	0.67		135 +++	115 +++	95 +++	115 +++		112.5
M. S.	M	Pneumonia	0.76	0.875	155 +++	165 +++	105 +++	185 +++		152.5
J. D.	M	Pneumococcus meningitis	0.80		225 0	255 0	195 0	175 0		212.5
J. F.	M	Pulmonary carcinoma with metastases	0.85	1.05	155 ++	155 +++	165 +++	165 ++		160.0
A. R.	M	Pneumonia	1.20		370 +	440 ++	280 ++	445 ++		383.8

\* The values in the two columns represent determinations on duplicate samples of plasma, handled differently, as described in the text.

† +++++, +++, ++, + and 0 represent different degrees of clot retraction.

the extent of retraction of a clot and its tensile strength. This is not entirely surprising if we visualize fibrin as a fibrous protein similar to fibroin, hair keratin, and muscle myosin, composed of polypeptide chains arranged roughly in parallel strands (6). If, during clot retraction, the polypeptide chain is conceived to shorten in accordion fashion due to an intra-molecular transformation, then the number of polypeptide chains in a unit cross-sectional area, upon which the tensile strength would depend, would not be affected.

Incidentally, the elastic properties of plasma clots are well illustrated by the fact that equal increments in the weight load produce approximately equal increases in the length of the clot. With normal specimens, the average increase in length with every 10 gram increment was approximately 0.2 cm. With regard to the ability of the clot to resume its original length when the weights are removed, we have observed this to be only partial. The greater the load, the more pronounced is the disparity between the original and



years). Specimens 1, 4, and 7 were from healthy young blood donors; 2, 3, and 10 were from healthy males who had undergone minor surgical procedures on the hospital ward; 5, 6, and 9 were from laboratory workers; 8 and 11 were from females studied in the Out-Patient Department and reported as free of organic disease.

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M. A.	M	Inguinal hernia	0.32		55 ..... 0	75 ..... 0	75 ..... +	35 ..... 0	65 ..... 0	61.0
E. B.	M	1. Possible typhoid fever 2. Possible trichinosis	0.40		45 ..... +	55 ..... ++	55 ..... ++	55 ..... 0		52.5
R. W.	F	Pulmonary tuberculosis, healed	0.40	0.44	65 ..... ++	55 ..... +++	75 ..... +++	75 ..... +++		67.5
J. P.	M	Myelogenous leukemia	0.42		95 ..... 0	45 ..... 0	95 ..... 0	95 ..... ++		80.0
M. A.	F	Pregnancy	0.43	0.48	115 ..... +++	65 ..... +++	145 ..... +++	100 ..... 0		106.2
A. W.	M	Nephritis	0.44	0.48	55 ..... +++	75 ..... +++	85 ..... +++	35 ..... +++		62.5
F. N.	M	Acute rheumatic fever	0.46	0.63	55 ..... ++	85 ..... +++	65 ..... ++	65 ..... 0		67.5
K.	F	Pregnancy	0.50	0.54	135 ..... +++	95 ..... 0	105 ..... 0	95 ..... 0		107.5
C. O.	F	Pulmonary tuberculosis	0.505	0.65	145 ..... +++	75 ..... +++	135 ..... +++	75 ..... +++		107.5
M. A.	M	Fracture of left femur	0.525		85 ..... +++	85 ..... +++	75 ..... +++			81.6

TABLE II—Continued

Patient	Sex	Diagnosis	Fibrinogen*		Tensile strength and clot retraction (quadruplicate determinations)†					Average tensile strength
					105	85	105	95		
L. L.	M	Adenocarcinoma, hepatic flexure	0.55		105 +++	85 ++++	105 +++	95 +++		97.5
S. S.	M	Acute rheumatic fever	0.56		175 +++	95 +++	155 +++	95 +++		130.0
A. A.	M	Inguinal hernia	0.57		75 ++	65 +++	75 +++	65 +++		70.0
M. C.	M	Carcinomatosis	0.585		165 +++	85 0	155 +++	145 +++		137.5
P. C.	M	Carcinoma of rectum	0.595	0.79	145 +++	65 0	85 ++++	105 +++	95	99.0
C. B.	F	Retroperitoneal malignancy	0.64		155 ++	85 +++	95 +++	105 ++		110.0
K. B.	M	Pulmonary carcinoma	0.675		155 +++	135 +++	85 +++	95 +++		117.5
K. D.	M	Cervical adenitis	0.67		135 +++	115 +++	95 +++	115 +++		112.5
M. S.	M	Pneumonia	0.76	0.875	155 +++	165 +++	105 +++	185 +++		152.5
J. D.	M	Pneumococcus meningitis	0.80		225 0	255 0	195 0	175 0		212.5
J. F.	M	Pulmonary carcinoma with metastases	0.85	1.05	155 ++	155 +++	165 +++	165 ++		160.0
A. R.	M	Pneumonia	1.20		370 +	440 ++	280 ++	445 ++		383.8

\* The values in the two columns represent determinations on duplicate samples of plasma, handled differently, as described in the text.

† +++++, +++, ++, + and 0 represent different degrees of clot retraction.

the extent of retraction of a clot and its tensile strength. This is not entirely surprising if we visualize fibrin as a fibrous protein similar to fibroin, hair keratin, and muscle myosin, composed of polypeptide chains arranged roughly in parallel strands (6). If, during clot retraction, the polypeptide chain is conceived to shorten in accordion fashion due to an intra-molecular transformation, then the number of polypeptide chains in a unit cross-sectional area, upon which the tensile strength would depend, would not be affected.

Incidentally, the elastic properties of plasma clots are well illustrated by the fact that equal increments in the weight load produce approximately equal increases in the length of the clot. With normal specimens, the average increase in length with every 10 gram increment was approximately 0.2 cm. With regard to the ability of the clot to resume its original length when the weights are removed, we have observed this to be only partial. The greater the load, the more pronounced is the disparity between the original and

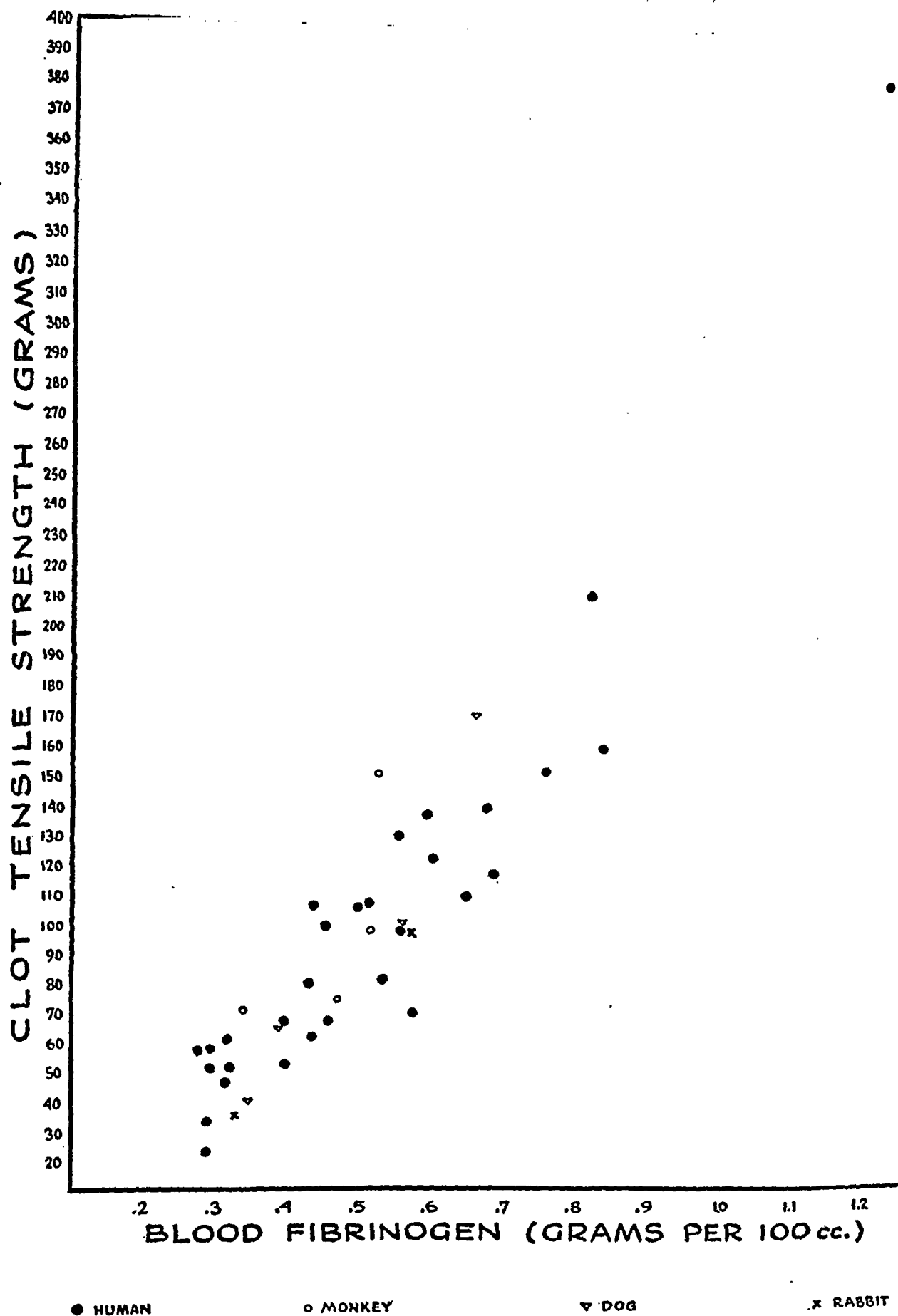


FIG. 2. RELATION OF CLOT TENSILE STRENGTH TO BLOOD FIBRINOGEN

# THE ELECTROPHORETIC ANALYSES OF THE SERUM PROTEINS IN DISEASES OF THE LIVER

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The intimate association of the liver with the maintenance of normal serum proteins is well established. An increase in serum globulin, with inversion of the albumin-globulin ratio, is a common finding in cirrhosis of the liver, but normal serum proteins are observed frequently in the acute parenchymatous diseases of the liver like catarrhal jaundice and arsenical hepatitis (1). The serum protein fractions in cancer of the liver are quite variable.

Determination of the serum proteins by the usual precipitation methods has led to the conception that the proteins may be considered normal when the serum albumin or globulin content falls within the empirically determined normal range. By electrophoretic analysis, however, the protein constituents within the serum globulin may be markedly abnormal, although their sum total by chemical analysis falls within the normal range.

Electrophoretic studies by Luetscher (2) and Longworth (3) indicate that an increase in beta and gamma globulin, associated with a decrease in albumin, occurs in cirrhosis of the liver. Only a few cases of liver cirrhosis were studied, and most of these investigations were made in the advanced stages of the disease, in which the serum proteins already appeared abnormal by the usual chemical methods. Little is known, however, about the electrophoretic distribution of these proteins in different types of liver disease and in various stages of hepatic insufficiency, particularly in the early stages of liver disease where the serum proteins are presumably normal by chemical analysis.

The present investigation includes the electrophoretic analysis of the serum proteins of patients with acute parenchymatous liver disease, cirrhosis of the liver, and cancer of the liver. Patients with extrahepatic jaundice caused by gall stones were also studied, and the results obtained by electrophoretic and chemical analysis in the various types of liver disease and in extrahepatic jaundice were

compared. Patients with varying degrees of liver insufficiency were studied in each group.

## METHOD

The blood samples were obtained from the patients before breakfast. The serum was diluted with 3 parts of veronal buffer made by mixture of the required amounts of 0.025 M veronal, 0.025 M HCl, and 0.025 M NaCl to a pH value of 7.8 at 25° C., and dialyzed against several liters of this buffer for 3 to 4 days, at 3° C., the buffer being changed daily. The protein solution was then centrifuged at 3° C. before being introduced into the electrophoresis cell. The electrophoretic experiments were carried out in the Tiselius apparatus (4) at 3° C., the electrophoretic patterns being recorded by the method described by Longworth (5).

The concentrations of the components of serum protein were estimated from the electrophoretic diagrams obtained from the descending boundaries.

## RESULTS

### Normal serum

The 4 protein fractions of human serum demonstrable by this method, as first noted by Tiselius (4) and Stenhagen (7), are albumin and

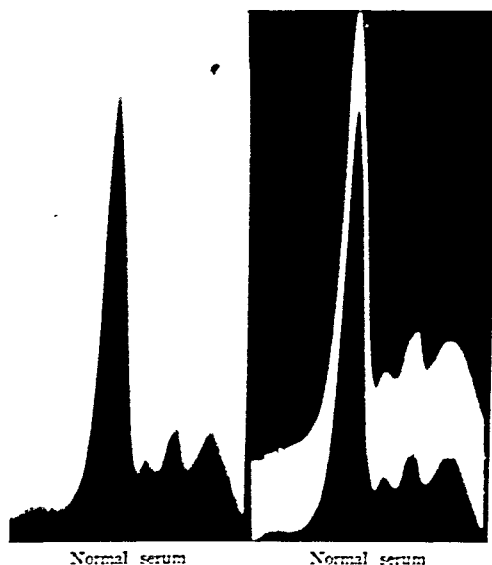


FIG. 1. NORMAL SERUM

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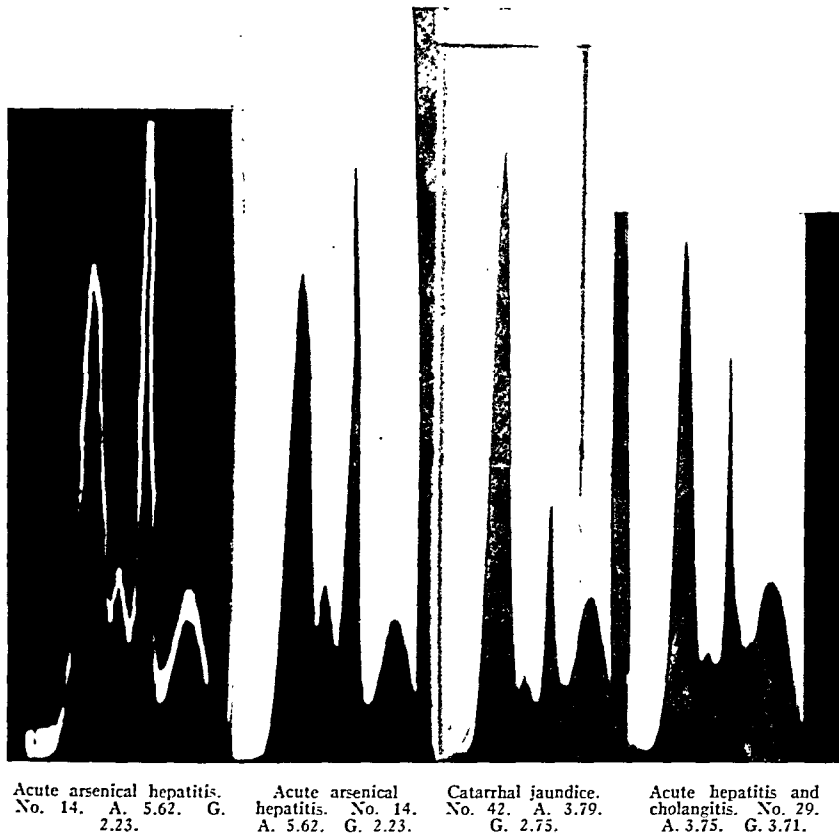


FIG. 2. ACUTE PARENCHYMATOUS DISEASES OF THE LIVER

or images. A fifth protein appears between the beta and gamma globulin in the anode, and the "delta effect" described by Longsworth may be seen in the very large gamma globulin of the ascending boundary. In the descending boundary,

Catarrhal jaundice. No. 32. A. 4.33. G. 2.45.

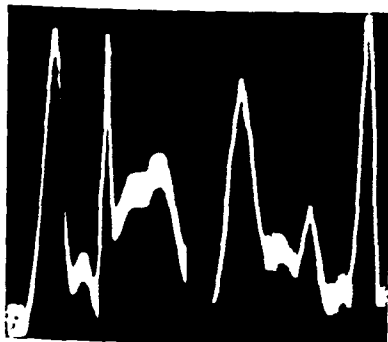


FIG. 3. CATARRHAL JAUNDICE

however, the additional protein appears to be a component of the gamma globulin, and the "delta effect" is not observed.

An increase in both beta and gamma globulin was observed in the serum of the patient with acute arsenical hepatitis (Table III, case 14). This 18-year-old patient became severely jaundiced following the third of a series of arsenical injections. The icteric index rose to 150, and the patient presented the symptoms of complete obstructive jaundice. At operation, no extrahepatic disease was found, and a biopsy of the liver revealed a severe hepatitis with intrahepatic obstruction, characterized by bile casts in the hepatic bile capillaries and edema of the periportal tissue.

Several chemical determinations of the serum proteins revealed a normal albumin-globulin ratio. The serum proteins were abnormal by electrophoretic analysis, however, the albumin being reduced to 44.5 per cent and the gamma globulin

alpha, beta, and gamma globulin, in the order of their electrophoretic mobility. There is excellent agreement among various workers concerning the proportions of these fractions in normal serum. In the 5 normal patients studied here (Figure 1), the serum albumin constituted 62.5 to 65.5 per cent of the total protein, alpha globulin, 6.2 to 7.9 per cent, beta globulin, 12.6 to 15.2 per cent, and gamma globulin, 13.1 to 15.7 per cent (Table I). The average normal values, found in this

TABLE I

*Percentage composition of serum proteins in normal serum*

Patient	Total protein	A/G ratio	Albumin	$\alpha$	$\beta$	$\gamma$
1	7.13	4.98/2.15	65.4	6.2	12.6	15.7
2	7.30	4.55/2.75	62.5	7.9	14.6	15.0
3	7.60	4.75/2.85	62.8	7.5	15.1	14.6
4	7.00	4.81/2.19	65.5	6.4	15.0	13.1
5	7.40		64.0	7.1	15.2	13.7

laboratory, of 64 per cent for serum albumin and 7.0 per cent, 14.5 per cent, and 14.4 per cent for the alpha, beta, and gamma globulins, respectively, closely approximate those reported by Svensson (6), Longworth (3), Luetscher (2), Gutman (8), and Kekwick (9) (Table II). The high

TABLE II

*Ratio of concentration of each globulin/albumin concentration for normal serum*

$\alpha$ /Albumin	$\beta$ /Albumin	$\gamma$ /Albumin	Reference
0.13	0.26	0.17	Svensson
0.12	0.23	0.20	Longworth
0.11	0.21	0.19	Luetscher
0.12	0.21	0.26	Gutman
0.08	0.19	0.43	Kekwick
0.11	0.23	0.22	Gray and Barron

value for gamma globulin reported by Kekwick is caused by the "delta effect," probably due to diffusion, observed in the ascending boundary. All of our studies were made in the descending boundary to obviate this effect, as suggested by Longworth and others.

#### *Acute parenchymatous liver disease*

The serum proteins of 5 patients with catarrhal jaundice, and 1 with an acute arsenical hepatitis, were studied electrophoretically (Table III). The catarrhal jaundice patients were between the ages of 5 and 25 and were rather severely jaundiced, with icteric indices between 21 and 40.

TABLE III

*Percentage composition of serum proteins in acute parenchymatous diseases of the liver*

Patient	A/G ratio	Al- bumin	$\alpha$	$\beta$	$\gamma$	Diagnosis
14	5.62/2.23	44.5	9.0	28.2	18.3	Acute arsenical hepatitis (biopsy)
29	8.75/3.71	42.8	5.8	20.5	30.9	Catarrhal jaundice (biopsy)
32	4.33/2.45	40.3	7.5	8.0	44.2	Catarrhal jaundice
33	3.16/3.14	48.8	9.1	25.9	16.2	Catarrhal jaundice
42	3.79/2.75	56.7	7.7	13.4	22.2	Catarrhal jaundice
68	3.91/2.25	58.1	6.0	10.1	25.8	Catarrhal jaundice

They presented the typical history and course of an acute infectious hepatitis; biopsy confirmed the diagnosis in 1 patient with catarrhal jaundice (Table III, case 29), and in one with acute arsenical hepatitis (Table III, case 14).

Although the total serum globulin determined chemically by the method of Campbell and Hanna (10) was normal in 3 of the 5 patients with catarrhal jaundice (Table III, cases 32, 42, 68), electrophoretic analysis revealed an abnormal increase in beta or gamma globulin or both in every instance (Figure 2). The increase of beta globulin to 20.5 and 25.9 per cent noted in 2 patients (Table III, cases 29 and 33) may be considered valid, since the blood cholesterol and the total fats which migrate with beta globulin were normal.

A considerable increase in gamma globulin, with values ranging between 22.2 and 44.2 per cent, was observed in 4 of the 5 cases of catarrhal jaundice. This increase in the gamma globulin to an average of 25.9 per cent and the concomitant decrease in serum albumin are the characteristic protein changes in the acute parenchymatous liver diseases.

That these protein abnormalities may be present in spite of a normal albumin-globulin ratio, determined chemically, is well illustrated in the case of the 5-year-old child with catarrhal jaundice (Table III, case 32). The albumin was decreased to 40.3 per cent and the gamma globulin increased to 3 times its normal value, although the albumin-globulin ratio obtained by the usual fractional precipitation methods remained normal (Figure 3).

Figure 3 also demonstrates the fact that the ascending and descending boundaries are not mir-

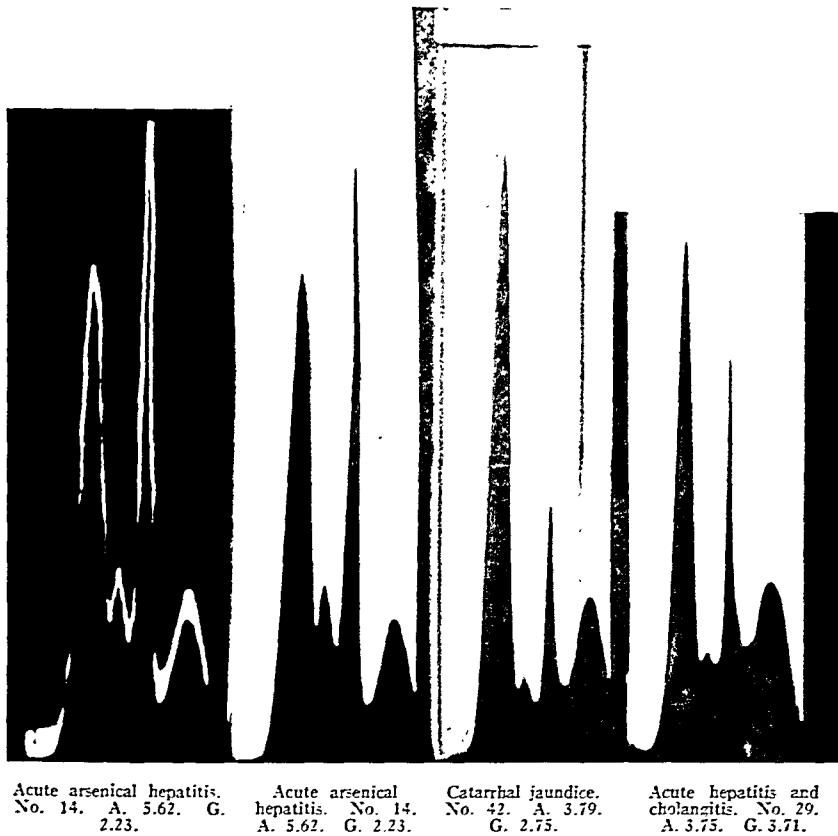


FIG. 2. ACUTE PARENCHYMATOUS DISEASES OF THE LIVER

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Catarrhal jaundice. No. 32. A. 4.33. G. 2.45.

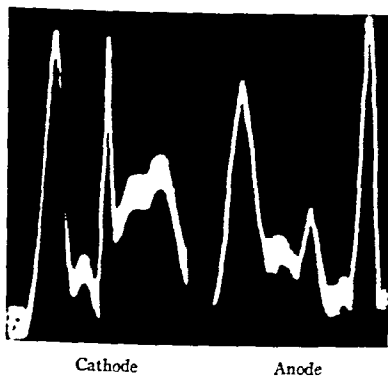


FIG. 3. CATARRHAL JAUNDICE



increased to 18.3 per cent (Figure 2). The large increase in beta globulin to 28.2 per cent may be attributed in part to the high serum cholesterol (714 mgm. per cent).

#### *Cirrhosis of the liver*

Electrophoretic studies of the serum proteins were made in 12 patients with cirrhosis of the liver (Figure 4). The diagnosis was verified in 8 of these cases by autopsy, biopsy, or peritoneoscopy. Atrophic cirrhosis of the liver was found in 6 cases, hypertrophic periportal cirrhosis in 2, polyserositis with cirrhosis of the liver in 1, and pellagra with atrophic cirrhosis in 1. These patients were all severely jaundiced with icteric indices between 30 and 120.

The abnormality of the serum proteins is more pronounced in cirrhosis of the liver than in any other form of liver disease. A decrease in serum albumin or increase in serum globulin, determined chemically, was observed in 9 of 10 cases

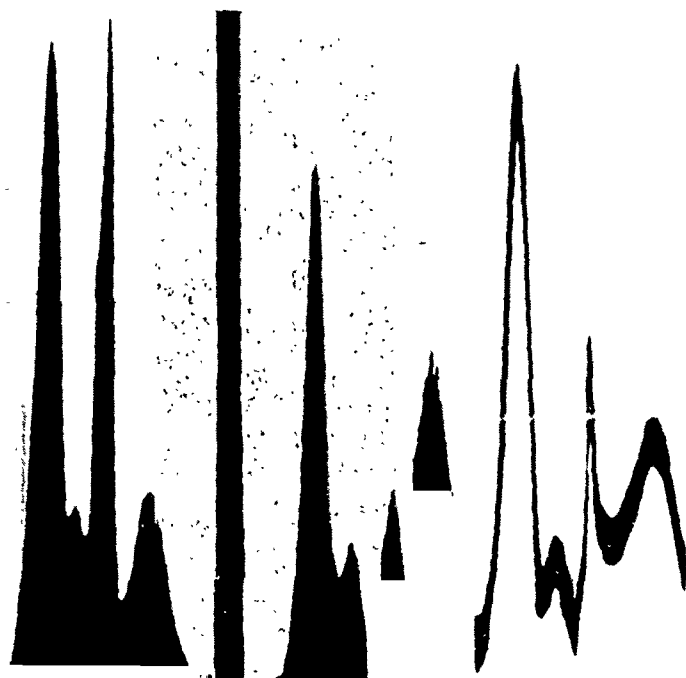
TABLE IV  
*Percentage composition of serum proteins in cirrhosis of the liver*

Patient	A/G ratio	Albumin	$\alpha$	$\beta$	$\gamma$	Diagnosis
7	3.60/3.04	52.8	8.6	22.5	16.1	Atrophic cirrhosis (autopsy)
15	4.77/3.22	45.4	4.8	30.7	19.1	Xanthomatosis and biliary cirrhosis (biopsy)
20	5.11/2.54	64.5	3.5	18.4	13.6	Atrophic cirrhosis
22	3.06/3.45	36.0	5.1	14.5	44.4	Atrophic periportal cirrhosis (autopsy)
25	1.98/2.90	38.1	10.5	20.6	30.8	Atrophic cirrhosis (peritoneoscopy)
28	3.12/2.29	58.8	9.1	13.6	18.5	Hypertrophic periportal cirrhosis (autopsy)
31	4.37/3.13	64.3	4.6	9.8	21.3	Pellagra and atrophic cirrhosis
35	2.94/4.41	42.7	10.1	12.0	34.2	Atrophic cirrhosis
36		39.9	11.7	9.2	39.2	Polyserositis and capsular cirrhosis (autopsy)
47	2.63/3.92	39.6	9.8	14.4	36.2	Atrophic cirrhosis (autopsy)
61	3.23/6.02	35.1	4.5	11.4	49.0	Hemochromatosis and cirrhosis
62		31.6	10.0	28.5	29.9	Hypertrophic cirrhosis (autopsy)

(Table IV). Electrophoretic studies demonstrate even more severe alteration of the serum proteins. Serum albumin was decreased to an average of 45.7 per cent and varied from 31.6 to 64.5 per cent. These determinations were only slightly lower in the 6 cases with ascites than in the 6 without ascites, the average for the former group being 44.7 per cent and for the latter, 46.8 per cent. As many albumin determinations of 31.6 to 39.9 per cent were observed in patients without ascites as in those with ascites, although the 2 normal values of 64.3 and 64.5 per cent occurred in patients without ascites.

The most consistent and characteristic globulin alteration is a large increase in the gamma globulin. This was observed in 11 of the 12 cases studied. The gamma globulin was increased to an average of 29.3 per cent, a 100 per cent increase over the normal. Determinations as high as 36.2, 39.2, 44.4, and 49.0 per cent were observed in this group.

Although the beta globulin was increased in 5 of the 12 patients studied, high blood cholesterol values were found in 2 of these cases (Table IV, cases 15 and 62). In the remaining 3 cases in which the blood cholesterol and fat were normal,



Xanthomatosis and biliary hepatic cirrhosis. A. 4.53. G. 2.37. No. 15.  
Cirrhosis of the liver. A. 2.94. G. 4.41. No. 35.  
Hepatic cirrhosis and ascites. A. 4.37. G. 3.13. No. 31.

FIG. 4. CIRRHOSIS OF THE LIVER

the beta globulin constituted 18.4 to 22.5 per cent of the total protein. In general, the average beta globulin for the 10 patients with normal blood cholesterol and fat determinations was normal (14.6 per cent), although moderate increases in the beta globulin were noted in several cases (Table IV, cases 7, 20, 25).

Abnormally high values of 9.8 per cent or more for alpha globulin were observed in 5 cases in which the albumin determinations were particularly low (31.6, 38.1, 39.9, 39.6, 42.7 per cent), and where there was an inadequate compensatory increase in beta and gamma globulin. In other instances with equally low serum albumin and normal alpha globulin values, there were unusually large compensatory increases in beta or gamma globulin (Table IV, cases 22, 61).

#### *Cancer of the liver*

The protein fractions were analyzed electrophoretically in 7 cases of metastatic carcinoma of the liver (Figure 5). The primary source of the carcinoma was the pancreas in 5 cases (Table V, cases 37, 54, 58, 60, 64), the stomach in 1 (Table V, case 57), and the rectum in 1 (Table V, case

TABLE V  
*Percentage composition of serum proteins in metastatic carcinoma of the liver*

Patient	A/G ratio	Al- bumin	$\alpha$	$\beta$	$\gamma$	Primary source of liver metastases
37	5.42/3.56	52.7	8.6	24.2	14.5	Pancreas (biopsy of liver)
45	3.59/3.99	45.3	6.9	18.6	28.2	Rectum (autopsy)
54	5.62/2.76	60.6	8.0	19.7	11.7	Pancreas
57		40.0	8.6	29.2	22.2	Stomach (autopsy)
58	4.15/2.36	39.5	4.7	18.4	37.4	Pancreas (biopsy of liver)
60		58.6	9.1	20.4	11.9	Pancreas
64	3.55/2.26	44.7	8.7	30.1	16.5	Pancreas (biopsy of liver)

45). The diagnosis was confirmed by autopsy or biopsy in 5 of the 7 patients. Jaundice was quite pronounced in each instance, the icteric index varying between 40 and 200.

The serum albumin-globulin ratio, determined by the fractional precipitation method, was normal in 2 of the 5 cases studied. Electrophoretically, however, the albumin was moderately decreased in 6 of the 7 cases, and varied between 39.5 and 60.6 per cent, with an average of 48.8 per cent.

Abnormalities of the serum globulins are less prominent in secondary carcinoma of the liver

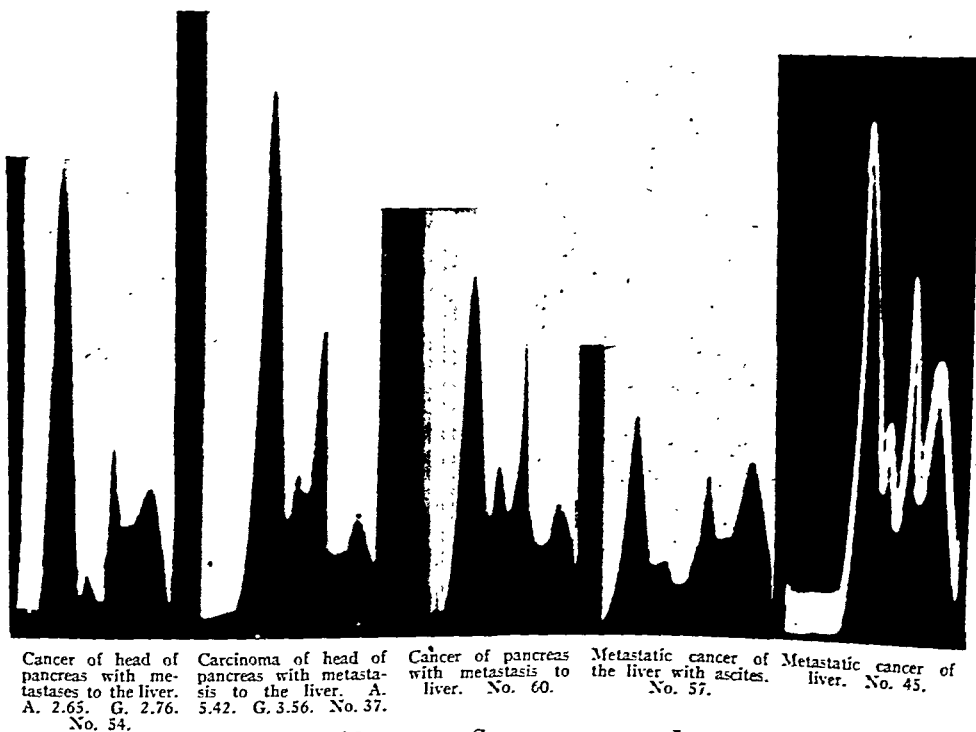


FIG. 5. METASTATIC CARCINOMA OF THE LIVER

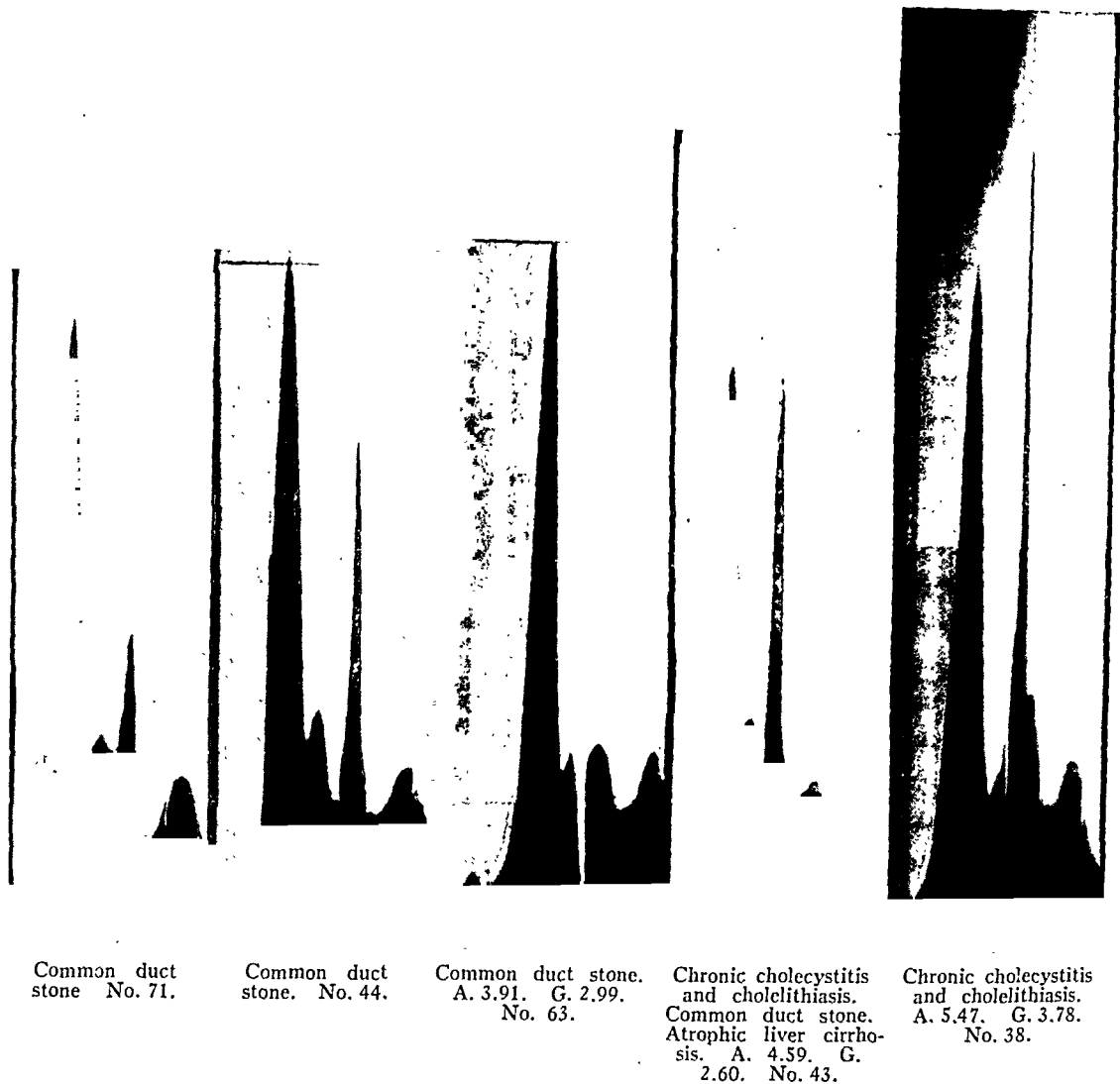


FIG. 6. EXTRAHEPATIC JAUNDICE

than in any other form of intrahepatic disease. The gamma globulin was essentially normal in 4 of the 7 cases, and was increased in the 3 remaining cases. The increase in beta globulin noted in all 7 cases may be explained, in part, at least, by the high blood cholesterol values which were present in the 5 cases with carcinoma of the head of the pancreas. The beta globulin was moderately elevated, however, in 1 case (Table V, case 45) in which the blood cholesterol was normal.

*Common duct obstruction*

Jaundice alone does not produce significant alteration of the serum proteins (Figure 6). This fact was demonstrated in 5 patients with a common duct stone, whose livers appeared grossly normal on surgical exploration. The jaundice was quite severe in all cases, as illustrated by icteric indices of 30, 32, 68, 93, and 125.

Although the serum albumin was decreased to 50.3 and 53.6 per cent in 2 cases, the gamma globulin was essentially normal in every case. It is interesting to observe that the beta globulin was normal in 2 cases, slightly elevated in 2 cases, and increased to 25.8 per cent in 1 case, although the serum cholesterol determinations were greatly increased in all 5 cases (250 to 416 mgm. per cent). (Table VI.)

Case No. 38 deserves special mention. There

TABLE VI  
Percentage composition of serum proteins in common duct stone

Patient	Total protein	A/G ratio	Al- bumin	$\alpha$	$\beta$	$\gamma$
38	9.25	5.47/3.78	50.3	8.6	25.8	15.3
43	7.19	4.59/2.60	66.7	8.1	18.3	6.9
44			53.6	9.1	19.6	17.6
63	6.90	3.91/2.99	60.7	7.6	14.8	15.9
71	6.86	4.24/2.62	64.6	7.6	14.6	13.2

was in this patient an increase in the total protein concentration of the serum (9.25 per cent). The liver was enlarged; the icterus index was 68.1; the cholesterol content was 250 mgm. per cent. Whether the large increase in  $\beta$  globulin (25.8 per cent) could be attributed to the increased cholesterol, as Longsworth seems to think, was not established. However, the patient when operated for cholelithiasis and cholecystitis (many gall stones were found in the gall bladder) showed in the liver focal areas of necrosis and pyknosis of the nuclei of liver cells. Judging by analogy with the other hepatitis, one is tempted to conclude that the increased beta globulin was a manifestation of the hepatic disorder, confirmed by the microscopic examination.

*Albumin-globulin ratios obtained by electrophoretic separation of serum proteins and by fractional precipitation*

Electrophoretic analyses of the serum proteins yield lower albumin and higher globulin determinations, and consequently lower albumin-globulin ratios, than are obtained by fractional precipitation (Table VII), although occasionally the results may be identical by both methods.

It should be emphasized that the albumin-globulin ratio or the serum globulin may appear normal as determined by precipitation but definitely abnormal in the distribution of the globulin fractions by electrophoretic analysis. This is most often observed in the acute parenchymatous diseases of the liver, in which the serum proteins are usually normal when determined by precipitation, but markedly abnormal on electrophoretic analysis. It is in these early acute stages of liver disease that these alterations of the globulin fractions are most frequently demonstrated electrophoretically, in spite of normal serum protein determinations on chemical analysis. This was clearly demonstrated in 3 cases of catarrhal jaundice (Table III, cases 32, 42, 68) and in 1 case of arsenical hepatitis (Table III, case 14), in which the serum globulin or the albumin-globulin ratio was normal by chemical analysis, while the globulin distribution was so abnormal electrophoretically that gamma globulin increases of more than 100 per cent were observed.

Similar but less frequent examples of abnormal

TABLE VII

*Albumin-globulin ratios obtained by electrophoretic separation of serum proteins and by fractional precipitation*

	Albumin Globulin ratio	
	Electrophoresis	Fractional precipitation
Normal	1.89	2.31
Normal	1.90	2.19
Normal	1.67	1.65
Normal	1.69	1.67
Acute hepatitis and cholangitis (biopsy)	0.75	1.01
Catarrhal jaundice	0.95	1.00
Catarrhal jaundice	0.67	1.76
Catarrhal jaundice	1.30	1.38
Catarrhal jaundice	1.38	1.73
Arsenical hepatitis (biopsy)	0.81	2.52
Cirrhosis of liver with ascites (biopsy)	0.65	0.67
Diabetes mellitus and cirrhosis of liver	0.54	0.54
Atrophic cirrhosis of liver (autopsy)	0.56	0.88
Xanthomatosis and biliary cirrhosis (biopsy)	0.83	1.48
Hypertrophic cirrhosis of liver (autopsy)	1.42	1.36
Carcinoma of pancreas with metastases to liver (biopsy)	1.12	1.52
Carcinoma of rectum with metastases to liver (autopsy)	0.86	0.90
Carcinoma of pancreas with metastases to liver (biopsy)	0.65	1.75
Carcinoma of pancreas with metastases to liver (biopsy)	0.81	1.57
Common duct stone	1.98	1.99
Common duct stone	1.72	2.43

globulin distribution with normal albumin-globulin ratios were seen in cirrhosis of the liver (Table IV, case 20) and in metastatic carcinoma of the liver (Table V, case 58).

The differences in the albumin-globulin ratios obtained electrophoretically and by fractional precipitation are least discernable in cirrhosis of the liver. Here, the serum proteins are so definitely abnormal on chemical analysis that the albumin-globulin ratios determined by both methods approximate each other closely (Table VII). In metastatic carcinoma of the liver, however, the albumin-globulin ratios obtained electrophoretically are more abnormal than is suggested by the chemical determinations, while the A/G ratios in common duct stone are essentially the same by both methods of analysis, and are identical with the control studies.

## DISCUSSION

These studies indicate that the diseased liver is unable to produce albumin as readily as the normal liver, and the more severe the hepatic insufficiency, the greater is the impairment of albumin production. Consequently, in cirrhosis of the liver with severe diffuse hepatic damage, the protein abnormalities are more pronounced than in any other form of liver disease (Table IV). Albumin values below 40 per cent were observed in 6 cases of cirrhosis of the liver in the late stages of hepatic insufficiency, demonstrating the relationship of severity of disease to impairment of albumin production. The effect of external protein loss will be discussed later. In general, the mean serum albumin was lower in cirrhosis of the liver than in any of the other liver diseases.

Although the degree of liver damage and hepatic insufficiency was considerably less in the patients with acute parenchymatous hepatic involvement, marked impairment of albumin production was observed (Table III). The mean serum albumin determination was 48.5 per cent and values as low as 40.3 and 42.8 per cent were noted, although many of these patients were seen in the early stages of the disease, and none had ascites or albuminuria.

Extensive carcinomatous involvement of the liver caused impaired albumin production, depending upon the degree of liver insufficiency. The lowest albumin value of 39.5 per cent was seen in a patient with extensive carcinomatosis of the liver, without ascites, observed on surgical exploratory examination (Table V, case 58). Values below 50 per cent were observed in 3 other cases, in 1 of which, ascites was present (Table V, case 57). The albumin determinations were not uniformly low in this group, however, as may be noted by such values as 52.7, 58.6, and 60.6 per cent in cases with less extensive liver involvement.

The serum albumin determinations were essentially normal in 3 of the 5 cases of extrahepatic jaundice, although some impairment of albumin production, to 50.3 and 53.6 per cent, was seen in 2 cases. Prolonged jaundice alone may cause early liver damage, and the decreased values may reflect these changes.

It would appear from these observations that

the serum protein changes in liver disease result primarily from the inability of the liver to produce normal serum proteins, rather than to external loss of protein to the ascitic fluid. This is in agreement with the observations of several investigators. The albumin-globulin abnormalities may be as severe in the absence of ascites as in cases with massive ascites. This may be confirmed by comparing albumin values of 40.3, 42.8, 44.5, and 39.5, observed in patients with liver disease without ascites, to albumin values of 52.8, 58.8, 42.7, 39.9 and 39.6, noted in patients with ascites. In the 12 patients with cirrhosis of the liver, 6 had ascites and 6 did not. The mean albumin determination was 44.7 per cent in the former group, and 46.8 per cent in the latter, with as many albumin determinations below 40 per cent in one group as in the other. It must be concluded from these studies that although the protein abnormalities may be slightly more pronounced in the patients with ascites than without it, the abnormal protein determinations result primarily from the liver insufficiency itself.

The more severe the impairment of albumin formation in the liver, the greater is the attempt by the body to compensate with an increased output of beta and gamma globulins, especially the latter. Consequently, in cirrhosis of the liver, where the albumin values are lowest, the gamma globulin determinations are highest, constituting 44.4 and 49.0 per cent of the total protein in some cases, and averaging 29.3 per cent more than twice the normal gamma globulin value.

The most consistent and characteristic globulin abnormality in liver disease is the increase in the largest molecular weight fraction, the gamma globulin, and the impaired production of the smallest molecular weight protein, the albumin. These changes are seen most frequently, and to the greatest degree, in cirrhosis of the liver, and next most frequently, in the acute parenchymatous diseases in which the gamma globulin determinations of 44.2 and 30.9 per cent were observed; although the average for this group was 25.9 per cent. The least pronounced gamma globulin abnormalities occurred in cancer of the liver. The gamma globulin values were all normal in extrahepatic jaundice.

The increased beta globulin values are difficult to interpret since the blood lipoids migrate with

the beta globulin. It should be mentioned, however, that in 5 patients with common duct obstruction and high blood cholesterol determinations between 250 and 416 mgm. per cent, the beta globulin was elevated appreciably in only 1 case and to a lesser degree in 2 others. Significant increases in beta globulin (*i.e.*, a beta globulin increase in the presence of normal blood cholesterol) were observed in all the types of liver disease studied, but to a considerably less degree and frequency than the gamma globulin changes. Beta globulin values of 18.4, 20.6, and 22.5 per cent were observed in cirrhosis of the liver, 20.5 and 25.9 per cent in the acute parenchymatous diseases, and 18.6 per cent in metastatic carcinoma of the liver. Disregarding high blood cholesterol determinations, elevated beta globulin values were observed in 5 of the 12 cases of cirrhosis of the liver, 3 of 6 cases of acute parenchymatous liver disease, and in all 7 cases of metastatic carcinoma of the liver. In the latter, the blood cholesterol values were exceedingly high, varying between 340 and 600 mgm. per cent, since the malignancy of the pancreas caused complete long-standing biliary obstruction in 5 of the 7 cases.

It is interesting to observe that in several cases in which the serum albumin was very low, (below 40 per cent), and the beta and gamma globulin did not sufficiently compensate for the decrease in albumin, the alpha globulin was increased to 9.8 per cent or more (Table IV, cases 25, 35, 36, 47, 62), indicating that in extreme cases where the liver does not adequately compensate by producing an increase in the larger beta and gamma globulin fractions, it may then produce an increase of the smaller alpha globulin as well.

Abnormalities of the serum globulins are less prominent in metastatic carcinoma of the liver than in any other form of intrahepatic disease. This may be explained by the fact that in this disease there are areas of cancer tissue dispersed through and surrounded by normal liver tissue, and since the reserve of the liver is very large, the serum proteins are not appreciably altered until the very late stages of the disease.

Jaundice alone causes little change in the serum proteins, as demonstrated in the 5 patients with common duct stone. Although the icteric indices were quite high in these patients, the serum globulins were essentially normal.

## CONCLUSIONS

1. Electrophoretic analyses of the serum proteins yield lower albumin and higher globulin determinations, and consequently lower albumin-globulin ratios, than are obtained by fractional precipitation.

2. The distribution of the serum globulin fractions may be definitely abnormal electrophoretically in spite of a normal albumin-globulin ratio on chemical analysis.

3. An abnormality of two or more protein fractions was observed in every case of liver disease studied. The degree of abnormality depended on the severity of the disease.

4. The most characteristic alteration of the serum proteins in liver disease is a large increase in the gamma globulin and a decrease in serum albumin. These changes are seen most frequently and to the greatest degree in cirrhosis of the liver and next most frequently in the acute parenchymatous diseases.

5. Significant increases in beta globulin were observed in all types of liver disease, but to a considerably lesser degree and frequency than the gamma globulin changes.

6. Abnormalities of the serum proteins are less prominent in metastatic carcinoma of the liver than in any other form of liver disease.

7. The serum protein changes in liver disease result primarily from the inability of the liver to produce normal serum proteins, rather than from external loss in the ascitic fluid.

8. Jaundice alone does not produce significant serum protein changes.

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# URIC ACID CLEARANCE IN NORMAL PREGNANCY AND PRE-ECLAMPSIA<sup>1</sup>

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A rise in blood uric acid without nitrogen retention is the most consistent blood chemical finding in pre-eclampsia and eclampsia (1, 2). It is not known whether this hyperuricemia is of renal or metabolic origin. A disturbance of uric acid metabolism in the liver has been suggested (1). The idea that it may result from an elevated blood lactic acid has been shown to be untenable (3). Its possible relation to the hypertension in the disease has been mentioned, since a similar hyperuricemia has been reported in essential hypertension (4) and has been obtained experimentally (5) with the kidney pressor substance, renin.

Previous evidence has not indicated that a reduced uric acid excretion is of particular importance as a cause of the hyperuricemia. Cadden and Stander (1) found that the uric acid excreted daily in eclampsia was not essentially different from the normal. Nayar (6) reported a 13 per cent reduction in the uric acid clearance in eclampsia, but this was associated with a 61 per cent rise in the blood uric acid. However, recent reports (7 to 9) are in agreement that the glomerular filtration is reduced in these pregnancy toxemias. For this reason, we have investigated the uric acid clearance in normal pregnancy and in pre-eclampsia to determine its role in the hyperuricemia of the disease.

## METHODS

Ten patients with the clinical diagnosis of pre-eclampsia, and 12 who were clinically normal, were the subjects of this study. Three other patients with unclassified pregnancy toxemia were also studied. Uric acid clearances were done on all patients in the last trimester, as close to term as possible. In 4 pre-eclamptic, 3 unclassified, and 4 normal patients, the clearance was repeated at about 8 days postpartum. Most of the patients had renal function studied by simultaneous inulin, diodrast, and urea clearances, which were reported elsewhere (9).

All patients were well hydrated in order to obtain a good urine flow.<sup>2</sup> They were given a liter of water to drink at bedtime the night before the clearance was to be determined, and 200 cc. at 5:00, 5:30, and 6:00 a.m. At 6:20 a.m., a constant intravenous infusion of the inulin and diodrast solution was started and continued to the end of the test. At 7:00 a.m., the first clearance was begun after emptying the bladder by injecting 50 to 100 cc. of air, 3 times. The starting time was taken after the last air injection. Usually, 3 urine collections of 15 to 30 minutes duration were made. When the urine flow was below 2 cc. per minute, the urine was collected for periods up to 60 minutes. The bladder was emptied with air at the end of each urine collection. A blood sample was taken midway in both the first and third urine collections.

All clearances were calculated using the maximum clearance formula with plasma values (except urea where whole blood values were used) and were corrected to a body surface area of 1.73 sq. M. The reported clearances were generally averages from 2 or 3 clearance periods taken on one day. In the case of uric acid, no clearance was averaged when the urine flow was below 1 cc. per minute, the augmentation limit assumed for uric acid by Bröchner-Mortensen (10). With inulin, no clearance was averaged below a urine flow of about 1.1 cc. per minute.<sup>3</sup> No urea clearance was averaged below a urine flow of 2 cc. per minute.

Plasma tungstic acid filtrates were prepared by adding 4.5 cc. of water to 1 cc. of plasma, followed by 0.5 cc. of 10 per cent sodium tungstate, and slowly adding 4 cc. of N/12 sulfuric acid. Uric acid was determined on this filtrate by the method of Folin (12, 13). Urine uric acid<sup>4</sup> was determined by the direct method of Folin (13).

<sup>2</sup> An 8 per cent mannitol infusion is recommended (8) as a diuretic for toxemia patients that give low urine flows. This was not used in the present study.

<sup>3</sup> In the present series of normal pregnant patients, this urine flow was about the limit below which the inulin clearance occasionally fell. Since Chasis (11) found no decrease in the inulin clearance at a urine flow as low as 0.6 cc. per minute in man, the present decreases were due to collection errors that occur especially in late pregnancy (8, 9).

<sup>4</sup> One of us (N. K. S.) will show elsewhere that practically all the uric acid in human urine, as determined by the direct Folin method, is true uric acid, as determined by the uricase method (14). The same has previously (2) been shown to be true for the blood uric acid in pre-eclampsia and eclampsia.

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TABLE I  
*Uric acid clearance in normal pregnancy*

Case	Age	Surface area	Gra-vida	Week of pregnancy	Blood pressure	Protein-urea	Edema	Plasma uric acid	Blood urea-N	Average urine flow*	Plasma clearances		Urea clearance
											Uric acid	Inulin	
	years	sq. M.			mm. Hg			mgm. per cent	mgm. per cent	cc. per minute	cc. per minute	cc. per minute	cc. per minute
ANTEPARTUM													
1A	34	1.63	6	40	115/78	0	0	4.40	7.8	0.94	28.3	90	
2A	30	1.68	2	38	106/70	0	0	4.43		2.82	23.1		
3A	37	1.70	2	36	110/72	0	0	3.82		2.36	19.8		
4A	32	1.63	1	39	120/80	0	0	3.93		2.47	33.2		
5A	35	1.65	4	40	118/68	0	+	3.51	7.4	4.32	29.8	121	126
6A	31	1.87	3	40	128/100	0	0	4.62	6.0	7.16	21.3	120	97.3
7A	34	1.66	1	32	120/70	0	0	2.89	7.5	2.12	43.8	150	95.5
8A	40	1.52	4	40	128/80	0	0	3.91	7.5	1.34	38.3		
9A	24	1.52	2	36	105/50	0	0	3.84	6.1	1.44	35.2	131	78.2
10A	25	1.60	2	32	120/70	0	0	2.67	9.9	4.94	43.3	115	125
11A	38	1.40	5	40	120/70	0	0	4.68	10.0	4.44	30.2	67	62.1
12A	32	1.60	3	40	120/75	0	0	3.23	8.0	4.44	36.7	144	88.0
Mean								3.83	7.8	3.23	31.9	117	91.3
POSTPARTUM													
5A	35	1.51	4	7†	130/80	0	0	3.98	12.3	5.22	34.8	171	83.4
6A	31	1.77	3	8	110/70	0	0	4.26	11.9	5.49	23.4	108	71.7
9A	24	1.46	2	9	120/70	0	0	4.08	11.8	6.49	32.3	158	119
13A	21	1.51	1	9	120/70	0	0	4.41	13.0	1.96	26.7	115	86.5
Mean								4.18	12.3	4.79	29.3	138	90.1

\* Average urine flow only for uric acid clearance.

† Days postpartum.

When the urine contained an appreciable amount of protein, the analysis was made after deproteinization with acetic acid and heat, or on a tungstic acid filtrate prepared by adding 3.5 cc. of water to 2 cc. of urine, followed by 0.5 cc. of 10 per cent sodium tungstate, and slowly adding 4 cc. of N/12 sulfuric acid.

Plasma and urine inulin were determined by the method of Alving, Rubin, and Miller (15). Urea was determined on whole blood filtrate by the manometric hypobromite method of Van Slyke and Kugel (16). Urine urea plus ammonia was similarly determined (17) without removing protein.

#### RESULTS

A summary of the clinical data and of the uric acid, inulin, and urea clearances is given in Table I for the normal patients and in Table II for the pre-eclamptic and unclassified groups. It is seen that the antepartum plasma uric acid was elevated in most of the pre-eclamptic cases and that the mean (5.29 mgm. per cent) was 38 per cent higher than the mean for the normal group (3.83 mgm. per cent). The uric acid clearance showed a decrease in the pre-eclamptic group commensurate

with the increase of blood uric acid. The antepartum normal clearance averaged 31.9 cc. per minute as compared with 21.9 cc. per minute for the pre-eclamptic group, a decrease of 31 per cent. Despite considerable variation of the values in both groups, both the decrease of the clearance and the elevation of blood uric acid in the pre-eclamptic group were statistically significant (Table III). Thus, the "significance of the difference of the means" was 2.5 for the uric acid clearance and 3.8 for the blood uric acid. The distribution of the uric acid clearance values is shown in graph form in Figure 1. It is apparent that the pre-eclamptic clearances are generally lower than the normal clearances at all urine flows.

Since pre-eclamptic patients usually begin to recover after delivery and show a gradual return of the blood uric acid to normal, the effects of the disease may also be shown by comparing their antepartum and postpartum values. In 4 pre-eclamptic patients, the average blood uric acid at about 10 days postpartum was 4.54 mgm. per cent

TABLE II

*Uric acid clearance in pre-eclampsia and unclassified pregnancy toxemia*

Case	Mild or severe	Age	Sur-face area	Gra-vida	Week of preg-nancy	Blood pressure	Protein-urea	Edema	Plasma uric acid	Blood urea-N	Average urine flow*	Plasma clearances		Urea clearance
												Uric acid	Inulin	
years sq. M.						mm. Hg			mgm. per cent	mgm. per cent	cc. per minute	cc. per minute	cc. per minute	cc. per minute
ANTEPARTUM PRE-ECLAMPSIA														
1B	M	25	1.69	4	26	150/94	+++	++	3.92		2.80	14.8		
2B	M	32	1.86	1	40	150/100	++	Tr.	5.54	11.9	3.98	23.3	96	56.1
3B	M	35	1.99	8	40	135/90	+++	Tr.	6.92		1.47	12.8	84	
4B	S	20	1.53	1	38	150/112	+++	++	4.23	8.6	3.26	18.8		38.7
5B	S	23	1.69	3	28	152/102	+++	+++	4.63	8.7	3.34	35.2		74.0
6B	S	34	1.66	1	37	160/100	Trace	0	3.95	7.3	2.98	36.7	150	108
7B	S	27	1.68	1	39	142/94	++	0	5.39		1.31	34.7	141	
8B	S	22	1.73	1	38	140/90	++++	Tr.	5.93	12.0	2.25	22.1	107	74.2
9B	S	31	1.64	2	40	140/90	++++	Tr.	7.12		1.01	12.9	43	
10B	S	22	1.73	1	36	150/100	++++	++	5.27		1.10	7.8	38	
Mean									5.29	9.7	2.35	21.9	94	70.2
Percentage difference from antepartum normal pregnancy									+38.1	+24.4	-27.2	-31.3	-19.7	-23.1

## POSTPARTUM PRE-ECLAMPSIA

2B	M	32	1.75	1	8†	140/90	Trace	0	3.81	9.6	4.47	22.2	122	67.5
6B	S	34	1.59	1	11	123/75	0	0	4.43	8.1	4.92	33.3	37	60.4
7B	S	27	1.59	1	10	130/95	Trace	0	4.45	12.5	5.46	31.7	95	66.9
9B	S	31	1.53	2	10	110/90	Trace	0	5.48	12.3	6.27	36.2	146	113
Mean									4.54	10.6	5.28	30.9	100	77.0

## ANTEPARTUM UNCLASSIFIED TOXEMIA

1C	22	1.52		2	38	135/90	+++	0	7.13		1.38	14.3	113	
2C	25	1.91		2	34	160/105	Trace	0	3.84		1.46	23.6	97	
3C	24	1.83		2	39	160/105	++++	Tr.	5.24	8.6	4.41	15.0	58	73.4
Mean									5.40	8.6	2.42	17.6	89	73.4

## POSTPARTUM UNCLASSIFIED TOXEMIA

1C	22	1.43		2	6†	140/90	+	0	3.27	9.6	2.99	37.4	129	91.6
2C	20	1.85		2	9	130/90	++	0	3.98	10.4	6.33	27.0	142	94.9
3C	24	1.78		2	6	140/90	Trace	0	4.64	10.4	9.22	20.4	160	51.0
Mean									3.96	10.1	6.18	28.3	144	79.2

\* Average urine flow only for uric acid clearance.

† Days postpartum

TABLE III

*Statistical summary of antepartum uric acid clearance and blood uric acid data*

		Number of cases	Range	Mean	$\sigma$	$\sigma_m$	Significance of difference of means
Plasma uric acid clearance	Normal pregnancy	12	19.8 to 43.8	31.9	7.67	2.22	2.5
	Pre-eclampsia	10	7.8 to 36.7	21.9	10.51	3.33	
Plasma uric acid	Normal pregnancy	12	2.67 to 4.68	3.83	0.628	0.182	3.8
	Pre-eclampsia	10	3.92 to 7.12	5.29	1.08	0.343	

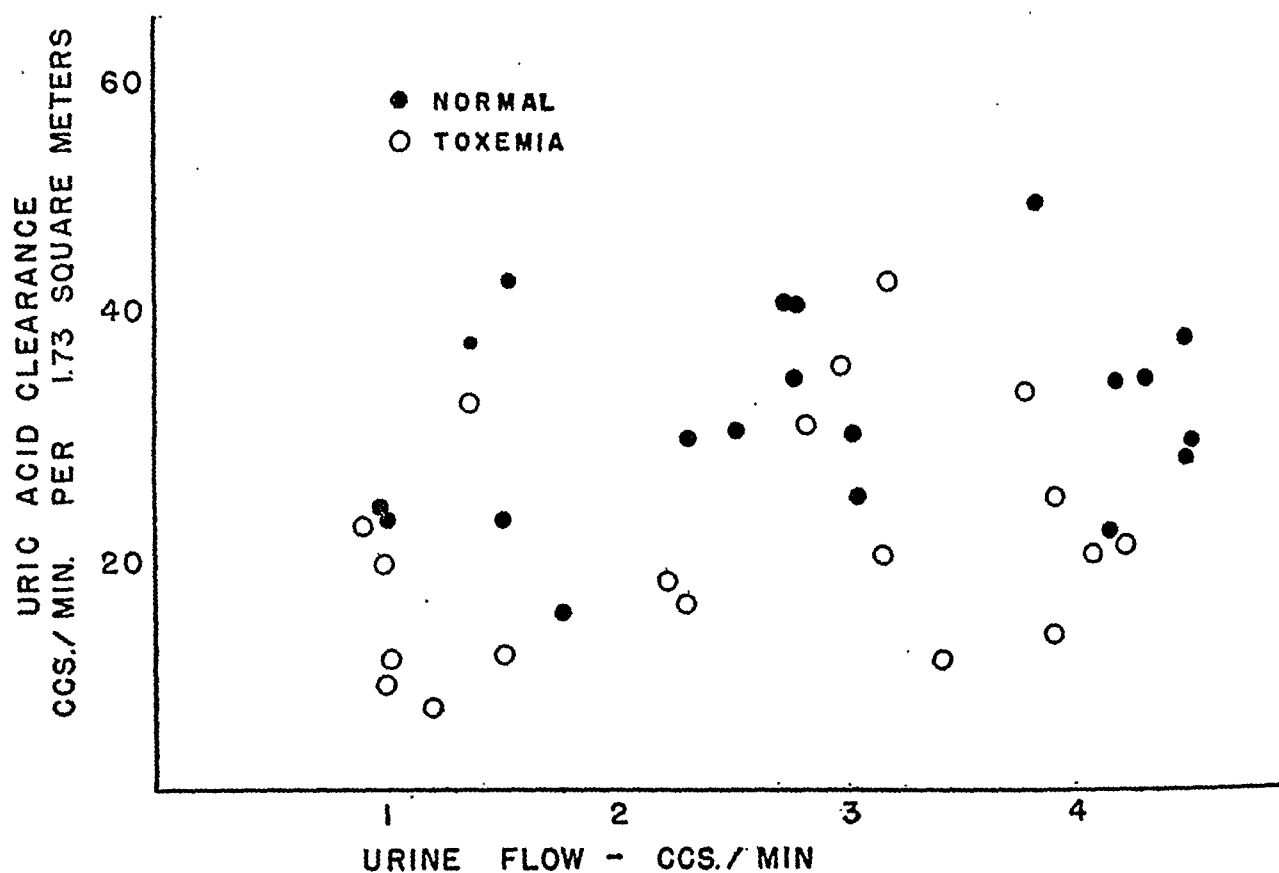


FIG. 1. DISTRIBUTION OF URIC ACID CLEARANCES IN ANTEPARTUM NORMAL PREGNANCY AND IN TOXEMIC PREGNANCY

as compared with their antepartum value of 5.50 mgm. per cent, or a rise of 21 per cent antepartum. The uric acid clearance postpartum was 30.9 cc. per minute and antepartum, 26.9 cc. per minute, or a decrease of 13 per cent antepartum. In 3 normal patients, the postpartum values differed by only a small percentage from their antepartum values.

One normal patient (case 7A) was of particular interest in that she subsequently developed severe pre-eclampsia (case 6B). The normal uric acid clearances determined in both the 31st and 33rd weeks of pregnancy were the same, 43.8 cc. per minute. The toxemic clearance in the 37th week of pregnancy was 36.7 cc. per minute, a decrease of 16 per cent. The blood uric acid rose 37 per cent. The postpartum changes showed a further decrease in the clearance with a rise in the blood uric acid.

The 3 unclassified toxemia patients also showed an antepartum hyperuricemia (+41 per cent) and decreased uric acid clearance (-45 per cent) relative to the normal group, and with corresponding changes of +36 and -38 per cent when compared with their postpartum values.

The inulin clearance, studied in 8 normal and 7 pre-eclamptic patients, was rendered 20 per cent in the toxemic group antepartum and rose postpartum. The urea clearance was reduced 23 per cent and the blood urea was increased 22 per cent in 6 cases of antepartum pre-eclampsia as compared with 7 normal cases.

#### DISCUSSION

It would appear from the finding of a decrease of about one-third in the uric acid clearance associated with a similar increase in the blood uric acid in pre-eclampsia, that a reduced clearance of this substance primarily accounts for the hyperuricemia. Additional evidence is given by the postpartum fall in blood uric acid and rise in the clearance in the toxemic group and by the changes found in one patient before and after becoming pre-eclamptic. Also consistent with this is that the 3 toxemic patients (cases 3B, 9B, 1C) with the highest blood uric acid values had a greater decrease in the uric acid clearance antepartum and the greatest rise postpartum (cases 8B, 1C). Their average blood uric acid was 7.06 mgm. per cent, or 84 per cent higher than normal, and their

average clearance was 13.3 cc. per minute, or 58 per cent lower than normal.

The data on the inulin clearance in pre-eclampsia agree with previous reports (7 to 9) of a diminished glomerular filtration in the disease and indicate the probable origin of the decreased uric acid and urea clearances. The reduction in the urea clearance is also in agreement with previous reports (18 to 20). The absence of an elevated blood urea (relative to the nonpregnant normal value) when the blood uric acid is raised in pre-eclampsia is apparently due to the fact that pregnancy normally lowers blood urea but not blood uric acid (21).

While Nayar (6) found a decreased uric acid clearance (— 13 per cent) in eclampsia, consistent with the present work, his failure to obtain a commensurate rise in blood uric acid (he found + 61 per cent) was probably due to several causes. Above all, the urine flows in his series (average in normal pregnancy = 0.56 cc. per minute, average in eclampsia = 0.74 cc. per minute) were at a level where collection errors are likely to occur (see footnote 3). Also he assumed that the augmentation limit for uric acid was the same as that for urea, 2 cc. per minute, whereas Brøchner-Mortensen (10) has shown that it generally lies between 0.5 cc. and 1 cc. per minute and has taken it to be 1 cc. per minute. Some difference in the blood uric acid between his and our series might be expected from the fact that his patients had eclampsia and ours had pre-eclampsia. For example, an elevated blood lactic acid from the eclamptic convulsions might temporarily raise the blood uric acid (3). However, the hyperuricemia of both pre-eclampsia and eclampsia is believed to be due fundamentally to the same cause.

#### SUMMARY

The average plasma uric acid clearance in 12 cases of antepartum normal pregnancy was 31.9 cc. per minute, and in 10 cases of antepartum pre-eclampsia, it was 21.9 cc. per minute, or a reduction of 31 per cent in pre-eclampsia. The average plasma uric acid in the corresponding groups was 3.83 and 5.29 mgm. per cent, or an increase of 38 per cent in pre-eclampsia. These changes, which are statistically significant, indicate that the hyperuricemia of pre-eclampsia (and eclampsia) may be due to a decrease in the uric acid clearance.

The inulin and urea clearances were reduced simultaneously with the uric acid clearance in pre-eclampsia, and indicate that the decreased uric acid and urea clearances were due to a reduction in glomerular filtration.

The absence of an elevated blood urea when the blood uric acid is raised in pre-eclampsia is apparently due to the fact that pregnancy normally lowers blood urea but not blood uric acid.

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# PNEUMOCOCCIC PNEUMONIA: THE PROGNOSTIC SIGNIFICANCE OF THE NUMBER OF PNEUMOCOCCI IN THE SPUTUM IN RELATION TO THERAPY, BACTEREMIA, TYPE, LEUKOCYTE COUNT, DURATION OF THE DISEASE, AGE, AND DEGREE OF INVOLVEMENT<sup>1</sup>

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A study of Wright-stained smears of rusty sputum from patients with pneumonia has revealed that the number of pneumococci per oil immersion field may be used as a reliable aid in prognosis (1). Furthermore, the specific response of individual patients to serum and to sulfonamides can be readily followed by examination of sputum at intervals during the course of the disease. While the end result of both of these forms of therapy is a reduction in the number of organisms in the sputum and in the lungs (2), this is accomplished in a different manner by each of the therapeutic agents. Adequate serum, in moderately ill patients, brings about a prompt agglutination of the pneumococci in the sputum within 6 to 12 hours. This is usually followed by phagocytosis and a gradual reduction in the number of organisms over a period of several days (3, 4). The sulfonamides, on the other hand, exert a direct bacteriostatic effect which is manifested by a reduction in the number of pneumococci within 12 to 36 hours. A striking decrease is obtained in the sputum, regardless of the number originally present, except in rare instances where drug-fastness develops (3, 5, 6). In this communication, the sputum counts will be compared as to prognostic significance with the time honored clinical and laboratory criteria, such as type, bacteremia, duration of disease, leukocyte count, age, and degree of involvement.

## METHOD

Specimens were obtained from patients on admission to the hospital and at 12-hour intervals throughout the period of production of rusty or bloody sputum. Thin smears were made directly from representative rusty portions and were immediately treated with Wright's blood

stain for 3 to 5 minutes.<sup>2</sup> The stain was flooded off without the addition of buffer and the slides were air dried. The capsules of the pneumococci were usually visualized as clear or occasionally as pink zones around the dark blue diplococci. The mucin stained pale pink and the leukocytes and red blood cells were differentiated as on a blood smear.

The necessity of obtaining good specimens of sputum directly from the patient cannot be overemphasized. Rusty sputum, relatively free of organisms other than pneumococci, gave the most consistent results. Satisfactory specimens were obtained from 90 per cent of the type I through VIII pneumonias. The remaining 10 per cent of the cases failed to produce rusty sputum and were, therefore, excluded from the study. Frankly purulent specimens contained large number of pneumococci which bore little relation to the clinical course or outcome of the pneumonia. Watery mucoid sputum usually showed so many contaminating mouth organisms and desquamated epithelial cells that they had to be discarded.

The average number of extracellular encapsulated pneumococci was determined by counting 10 widely separated fields on the slide. A microscopic field was considered suitable for counting if it contained 10 to 15 leukocytes or red blood cells, if organisms other than pneumococci were absent, and if the capsules of the pneumococci were distinctly visible. When good specimens of sputum were obtained, the counts increased or decreased progressively; sudden changes in the number per field were found to be primarily due to poor samples rather than to errors in technique.

By means of the foregoing method, the patients were classified according to the highest sputum count observed at any time during the production of rusty sputum. This count was used whether or not the number of organisms was subsequently reduced as a result of treatment. Although 24 patients showed an increase in sputum count during the period of hospitalization, the classification of these cases on the basis of their highest count did not materially affect the final results. Patients whose sputum counts did not exceed 10 per oil immersion field were classified in group A; those with 11 to 30 organisms were placed in group B; those with counts ranging from 31 to

<sup>1</sup> Supported by a grant from the Commonwealth Fund to the Michigan Department of Health Laboratories.

<sup>2</sup> Watery specimens were permitted to dry before the addition of stain.

75 were included in group C; and those with more than 75 pneumococci were placed in group D.<sup>3</sup>

### RESULTS

The following report is based upon 678 roentgenographically proved cases of pneumococcic pneumonia, admitted to the Detroit Receiving Hospital between January of 1938 and May of 1942. It includes all patients, except type III,<sup>4</sup> in whom the sputum was studied, irrespective of complications, length of hospitalization, and cause of death. Of the total cases studied, 78 per cent (groups A and B) were classified as mild to moderately severe, and 22 per cent (groups C and D) as severe to overwhelmed. Table I reveals the close correlation between the number of pneumococci per field in rusty sputum and the outcome of the pneumonia. The fatality rate, as shown in Table I, was only 2 per cent in group A, 9 per cent in group B, 30 per cent in group C, and 77 per cent in group D. These significant differences obtained, despite the fact that the majority of cases in group A received either supportive therapy or small doses of serum, whereas

TABLE I  
*Classification of cases by sputum groups*

Sputum group	Highest sputum count	Cases		Deaths	
		Number	Per cent of total	Number	Per cent fatality
A	10 or less	320	47	6	2
B	11 to 30	210	31	18	9
C	31 to 75	101	15	30	30
D	76 and over	47	7	36	77
Total		678	100	90	13

most of the cases in groups C and D received intensive chemotherapy, often supplemented by serum. Treatment was withheld from many patients in group A because one phase of this study was concerned with the evaluation of the sputum findings as a guide to specific therapy (7).

An analysis of the outcome in reference to ther-

<sup>3</sup> In previous publications, only 3 groups were listed, but the number of cases has now made possible the separation into 4. This change also necessitated a shift in the range of sputum counts within a given group in order to make the differences statistically significant.

<sup>4</sup> The type III cases will be considered separately.

apy is presented in Table II. The fatality rate in group A was as low with supportive therapy as it was with serum, sulfanilamide, and even with more modern chemotherapeutic agents. In group B, the clinical response to sulfapyridine, sulfathiazole, and sulfadiazine was usually more dramatic than with serum or sulfanilamide, but the final fatality rates of 9 and 7 per cent were essentially the same. The superiority of modern chemotherapeutic agents became evident in cases with more than 30 pneumococci per field. In group C, the fatality rate was 52 per cent with the older methods of treatment as compared with 20 per cent when sulfapyridine, sulfathiazole, or sulfadiazine was used. In group D, the corresponding figures were 94 per cent and 68 per cent. The use of serum in conjunction with the more recent chemotherapeutic agents was no more effective than chemotherapy alone in cases with sputum counts over 30 per field (Table II).

The prognostic significance of the type of pneumococcus has been stressed in the literature. In the present study, we obtained a fatality rate of 11 per cent for type I and 20 per cent for type II, which is representative of the trend in other published reports. A breakdown of these figures to permit a comparison of cases whose sputum counts fell within the same range (Table III) has shown that the fatality rates of type I and type II pneumonias were essentially the same as the composite figures for other types. Thus the fatality for cases with less than 30 pneumococci per field (groups A and B) was 5 per cent for type I, 4 per cent for type II, and 5 per cent for other types. The respective figures for cases with 31 to 75 pneumococci per field (group C) were 29 per cent, 33 per cent, and 30 per cent. The higher fatality rate for the entire group of 118 type II pneumonias may be explained by the fact that sputum counts above 30 were found in 32 per cent of the type II cases and in only 18 per cent of the type I cases. The results with types VII and VIII were similar to those obtained for type I. It is therefore apparent that the outcome of the pneumonia is more closely related to the sputum count than to the type of pneumococcus.

The unfavorable prognostic significance of bacteremia is universally recognized. Our fatality rate of 6 per cent among 453 cases with negative

TABLE II  
*The relation of therapy to sputum counts*

Therapy	Sputum group											
	A			B			C			D		
	Cases		Deaths	Cases		Deaths	Cases		Deaths	Cases		Deaths
	Number	Number	Per cent fatality	Number	Number	Per cent fatality	Number	Number	Per cent fatality	Number	Number	Per cent fatality
Supportive.....	75	1	1	2	0		1	1		3	3	
Serum.....	105	2	2	16	1	6	13	6		6	6	
Sulfanilamide.....	33	0		22	2	9	15	8		5	5	
Drug and serum*.....	6	0		2	0		2	1		2	1	
Total.....	219	3	1	42	3	7	31	16	52	16	15	94
Sulfapyridine.....	36	2		47	3	6	11	2		5	5	
Sulfathiazole.....	46	0		106	9	9	38	7	19	9	5	
Sulfadiazine.....	15	0		7	1		5	0		2	1	
Drug and serum*.....	4	1		8	2		16	5		15	10	
Total.....	101	3	3	168	15	9	70	14	20	31	21	68
Grand Total.....	320	6	2	210	18	9	101	30	30	47	36	77

\* The term drug and serum refers to serum plus sulfanilamide in contrast to serum plus sulfapyridine, sulfathiazole, or sulfadiazine.

blood cultures, as compared with 28 per cent among 225 cases with positive cultures, is representative of reports in the recent literature. A breakdown of these figures, however, according to sputum count (Table IV) revealed only minor differences in the fatality rates of bacteremias and non-bacteremias whose sputum counts fell within the same range. Thus, in group A, the fatality rate was 2 per cent with negative blood culture and only 5 per cent with positive blood culture. In group B, the death rate was 8 per cent among non-bacteremias as compared with 10 per cent among bacteremias. In group C, the correspond-

ing figures were 23 per cent and 34 per cent; in group D, 83 per cent and 76 per cent. The greater disparity in bacteremic and non-bacteremic fatality rates for the series as a whole may be largely explained by the fact that 45 per cent of the bacteremias and only 10 per cent of the non-bacteremias fell into groups C and D, which show a high fatality irrespective of blood culture. It is thus apparent that the outcome of pneumonia is correlated more closely with the number of pneumococci in the sputum than with the presence or absence of bacteremia.

Quantitative blood cultures were done in 165

TABLE III  
*The relation of type to sputum counts*

Sputum group	Type I			Type II			Other types		
	Cases		Deaths	Cases		Deaths	Cases		Deaths
	Number	Per cent of total	Per cent fatality	Number	Per cent of total	Per cent fatality	Number	Per cent of total	Per cent fatality
A	73	46	4	46	39	2	320	47	2
B	57	36	5	34	29	6	210	31	9
C	21	13	29	24	20	33	101	15	30
D	7	5	90	14	12	93	47	7	77
Totals	158	23	11	118	17	20	678	100	13



TABLE IV  
The relation of bacteremia to sputum counts

Sputum group	Blood culture negative				Blood culture positive				Totals		
	Cases		Deaths		Cases		Deaths		Cases		Deaths
	Num-ber	Per cent of total	Num-ber	Per cent fatality	Num-ber	Per cent of total	Num-ber	Per cent fatality	Num-ber	Per cent of total	Per cent fatality
A	276	61	4	2	44	20	2	5	320	47	2
B	131	29	10	8	79	35	8	10	210	31	9
C	40	9	9	23	61	27	21	34	101	15	30
D	6	1	5	83	41	18	31	76	47	7	77
Totals	453	67	28	6	225	33	62	28	678	100	13

of the 225 cases with bacteremia.<sup>5</sup> The fatality rate was 19 per cent among 137 cases with less than 50 pneumococci per cc. of blood. A breakdown of these cases according to sputum counts revealed a fatality of 8 per cent in 89 cases classed in groups A and B, as compared with a rate of 35 per cent in 48 patients classed in groups C and D. These data did not differ markedly from the 5 per cent fatality rate for the entire series of 530 cases falling into groups A and B, and the 44 per cent fatality rate for the entire series of 148 cases falling into groups C and D and offer further support to our contention that the outcome of pneumonia is correlated more closely with the number of pneumococci in the sputum than with the presence or absence of minor grades of bacteremia. The statistics for blood cultures exceeding 50 per cc. were inconclusive because of the small number of cases, but conformed with the generally accepted view that high grades of bacteremia have an unfavorable effect on prognosis. The fatality rate among 28 such cases was 75 per cent. A breakdown of these 28 cases according to sputum counts revealed 2 deaths (40 per cent) among 5 patients classed in groups A and B, and 21 deaths (91 per cent) among 23 cases in groups C and D. An analysis of the deaths showed that those in groups A and B were attributable chiefly to complications, whereas the majority in C and D were due to pneumonia *per se*.

In Table V, the results are analyzed according to the sputum count and the duration of the pneumonia prior to therapy. A study of this table reveals that the fatality rate was uniformly low

in group A and high in group D, regardless of the duration of the disease before the institution of therapy. The fatality rate in groups B and C was intermediate between A and D in both the early and late pneumonias, but tended to be lower when treatment was instituted prior to the fourth day. It should also be noted that the duration of the disease determined to some extent the group to which the patient belonged, since only 14 per cent of cases ill less than 48 hours fell into C and D, whereas 39 per cent of those with onsets of 144 hours and over were classed in these groups; however, the outcome of the pneumonia was correlated more closely with the sputum counts than with the duration of the disease prior to therapy.

The results in reference to sputum and leukocyte counts are recorded in Table VI. It is generally recognized that the prognosis is poor in pneumonias associated with white cell counts below 5,000 per c. mm. of blood. This was especially true before the introduction of modern chemotherapy. Our fatality rate of 42 per cent in 36 such cases, as compared with 10 per cent in 505 cases with leukocytosis, is representative of the trend in other published reports; however, a breakdown of the cases according to sputum counts showed a much closer correlation of the fatality rate with the number of pneumococci in the sputum than with the leukocyte count. There were no deaths among the 15 cases with leukopenia which showed less than 30 pneumococci per field whereas there were 15 deaths (71 per cent) among the 21 leukopenic cases which showed more than 30 organisms per field. The apparent discrepancy in the fatality rates for the entire series of cases of leukopenia and leukocytosis can be ex-

<sup>5</sup> We are indebted to Dr. Daniel Hasley for the data on quantitative blood cultures.

# PROGNOSTIC DATA IN PNEUMOCOCCIC PNEUMONIA

TABLE V  
The relation of duration of disease before therapy to sputum counts\*

Sputum group		TABLE V <i>The relation of duration of disease before therapy to sputum counts*</i>												STATISTICAL DATA																							
		Less than 48 hours						48 hours to 95 hours						96 hours to 143 hours						144 hours and over						Totals											
		Cases			Deaths			Cases			Deaths			Cases			Deaths			Cases			Deaths			Cases			Deaths			Cases			Deaths		
		Num-ber	Per cent of total	Per cent fatality	Num-ber	Per cent of total	Per cent fatality	Num-ber	Per cent of total	Per cent fatality	Num-ber	Per cent of total	Per cent fatality	Num-ber	Per cent of total	Per cent fatality	Num-ber	Per cent of total	Per cent fatality	Num-ber	Per cent of total	Per cent fatality	Num-ber	Per cent of total	Per cent fatality	Num-ber	Per cent of total	Per cent fatality	Num-ber	Per cent of total	Per cent fatality	Num-ber	Per cent of total	Per cent fatality			
A	115	54	4	4	4	109	47	2	2	6	24	79	9	131	13	29	34	231	8	17	8	231	8	17	8	231	8	17	8	231	8	17	8	231			
B	69	32	4	4	6	69	30	4	4	6	24	79	9	131	13	29	34	231	8	17	8	231	8	17	8	231	8	17	8	231	8	17	8	231			
C	22	10	3	3	14	34	15	8	15	7	8	89	10	11	11	6	60	13	20	20	15	20	15	20	15	20	15	20	15	20	15	20	15	20			
D	8	4	6	6	75	19	8	15	15	7	8	89	10	11	11	6	60	13	20	20	15	20	15	20	15	20	15	20	15	20	15	20	15	20			
Totals	214	32	17	8	231	8	231	34	29	13	29	131	13	29	131	13	29	131	13	29	131	13	29	131	13	29	131	13	29	131	13	29	131	13	29		

\* The time from the onset to admission to the hospital was used for the cases which received supportive therapy.

\* The time from the onset to admission to the hospital was used for the cases which received supportive therapy.

TABLE VI  
The relation of leukocyte counts to sputum counts

Sputum group		10,000 and over						5000 to 9999						Less than 5000						Totals					
		Cases			Deaths			Cases			Deaths			Cases			Deaths			Cases			Deaths		
		Num-ber	Per cent of total	Num-ber	Per cent fatality	Num-ber	Per cent of total	Num-ber	Per cent fatality	Num-ber	Per cent of total	Num-ber	Per cent fatality	Num-ber	Per cent of total	Num-ber	Per cent fatality	Num-ber	Per cent of total	Num-ber	Per cent of total	Num-ber	Per cent fatality		
																								Unknown	Cases
A	276	55	6	2	32	26	0	0	6	17	0	0	0	6	0	0	0	0	320	47	2	320	47	2	
B	158	31	15	9	41	34	3	7	9	25	0	0	0	2	2	0	0	0	210	31	9	210	31	9	
C	55	11	20	36	33	27	3	9	9	25	4	44	4	4	3	3	3	3	101	15	30	101	15	30	
D	16	3	8	50	16	13	14	88	12	33	11	92	3	3	3	3	3	3	47	7	77	47	7	77	
Totals	505	75	49	10	122	18	20	16	36	5	15	42	15	6	6	6	6	6	678	100	13	678	100	13	

TABLE VII  
*The relation of age to sputum counts*

Sputum group	Age 5 to 29 years				Age 30 to 54 years				Age 55 years and over				Totals		
	Cases		Deaths		Cases		Deaths		Cases		Deaths		Cases		Deaths
	Number	Per cent of total	Number	Per cent fatality	Number	Per cent of total	Number	Per cent fatality	Number	Per cent of total	Number	Per cent fatality	Number	Per cent of total	Per cent fatality
A	110	58	0	0	180	45	3	2	30	34	3	10	320	47	2
B	60	32	1	2	124	31	9	7	26	29	8	31	210	31	9
C	17	9	5	29	66	16	17	26	18	20	8	44	101	15	30
D	2	1	2	100	30	8	23	77	15	17	11	73	47	7	77
Totals	189	28	8	4	400	59	52	13	89	13	30	34	678	100	13

plained by the fact that 58 per cent of the former and only 14 per cent of the latter fell into groups C and D.

The relationship between the sputum counts and the age of the patients is shown in Table VII. A breakdown of the cases according to sputum groups revealed that the fatality rates correlated more closely with the number of pneumococci than they did with the factor of age. The progressive rise in fatality with age could be attributed in part to the distribution of cases within a given sputum group. Thus, 37 per cent of the oldest patients belonged to C and D whereas only 10 per cent of the youngest patients fell into the same groups. The patients aged 55 and over were of particular interest because the death rates of 10, 31, and 44 per cent were higher than the average values of 2, 9, and 30 per cent for groups A, B, and C, respectively. These discrepancies could be partially accounted for by the greater incidence of serious associated diseases such as cardiac failure, renal insufficiency, cirrhosis, and carcinoma in the group over 54 years of age.

In Table VIII, the results are analyzed according to sputum count and extent of the consolidation as revealed by physical examination and roentgenogram on admission to the hospital. When fatalities were calculated separately for cases with consolidation limited to one lobe and for those showing involvement of two or more lobes, a greater deviation from the average fatality within sputum groups was found than for any other factor studied. Thus, in group A, the fatality rate was 1 per cent when the pneumonia was confined to a single lobe and 8 per cent when it involved more than one lobe; in group B, the corresponding figures were 3 per cent and 22 per cent; in group C, they were 17 per cent and 47 per cent; and in group D, 56 per cent and 90 per cent. These data indicate that the degree of involvement before therapy was a significant factor in the outcome of the pneumonia irrespective of the sputum count. Extension of the process to additional lobes during the course of treatment occurred in only 13 per cent of the cases in groups A and B, and in 41 per cent of those in groups C and D.

TABLE VIII  
*The relation of involvement to sputum counts*

Sputum group	One or less lobes				More than one lobe				Totals		
	Cases		Deaths		Cases		Deaths		Cases		Deaths
	Number	Per cent of total	Number	Per cent fatality	Number	Per cent of total	Number	Per cent fatality	Number	Per cent of total	Per cent fatality
A	282	55	3	1	38	23	3	8	320	47	2
B	151	30	5	3	59	35	13	22	210	31	9
C	58	11	10	17	43	25	20	47	101	15	30
D	18	4	10	56	29	17	26	90	47	7	77
Totals	509	75	28	6	169	25	62	37	678	100	13

Sterile effusion, hepatitis, or purulent complications developed in 11 per cent of the cases in A and B, and in 32 per cent of those in C and D.

#### DISCUSSION

The correlation of the sputum counts with other established criteria of prognostic significance was undertaken with the expectation that all factors would contribute substantially to the final fatality rate in pneumonia. It was surprising to find that type, bacteremia, leukopenia, and late pneumonia were of minor importance when the cases were broken down according to the number of pneumococci in the sputum (Tables III, IV, V, and VI). These factors have gradually lost their prognostic significance with the advent of more effective forms of therapy. The increased fatality rates that still prevail in the presence of bacteremia leukopenia, etc., can be explained in part by their frequent association with high sputum counts. Thus, the fatality of 28 per cent among bacteremics was principally due to the fact that 45 per cent of the cases fell into the sputum groups C and D which accounted for 52 of the 62 deaths. On the other hand, the relatively low non-bacteremic fatality rate of 6 per cent was largely due to the fact that only 10 per cent of the patients fell into groups C and D. This small minority accounted for half of the non-bacteremic deaths, but their significance was statistically obscured by the remaining 90 per cent of the cases in groups A and B. Actually, the fatality rate was over three times as high among the 46 patients with negative blood cultures in groups C and D as that among the 123 cases with positive blood cultures in groups A and B. The differences in fatality among the common types of pneumococci could also be explained on the basis of the percentage distribution of cases in the various sputum groups. The abnormally high fatality rates in patients with types II and III pneumonia have, in the past, been responsible for the emphasis placed on type. The outcome in type III can be attributed, as shown in the following paper, to the capacity of this pneumococcus to form excessive amounts of capsular polysaccharide in the sputum and lungs, whereas the present study has shown that the fatality in type II pneumonia was due (Table III) to the greater proportion of cases with more than 30 pneumococci per field, as compared with other

types. The fact that the capsule of the type II pneumococcus is larger than any other except that of type III may be responsible for its ability to multiply in the lung and thus for the higher incidence of cases with sputum counts over 30. From a practical point of view, the outcome in type II, as in types other than III, depends primarily on the number of pneumococci in the sputum of a particular patient during the course of the pneumonia.

The results of the analysis have revealed that age and the degree of involvement were factors which significantly influenced the fatality rates within the four sputum groups. The increase in fatality with age was due chiefly to a corresponding rise in the incidence of groups C and D and partially to the degenerative diseases associated with old age. Some of the critically ill patients over 54 years of age produced a purulent sputum which never became rusty. These patients were unsuitable for study by the present method and it is entirely possible that they may ultimately be placed in a separate and distinct category. We were surprised to find that the extent of consolidation was next in importance to sputum count in determining the outcome of the pneumonia. When fatality rates were calculated by sputum groups, significant differences were noted between the cases with consolidation limited to one lobe and those with involvement of two or more lobes. These findings suggest that the outcome of pneumonia may be even more closely related to the total number of pneumococci in the two lungs than to their concentration in rusty sputum. There is some evidence to support this view since autopsy studies of fulminating cases have shown that pneumococci are distributed fairly uniformly throughout areas of gray hepatization, red hepatization, and inflammatory edema (2). Therefore, one might expect to find more pneumococci in the lungs with multiple than with single lobe involvement even though the sputum counts are the same. Another factor which may contribute toward the higher fatality rate is the degree of anoxemia associated with extensive pneumonia.

The utilization of the sputum as a method of prognosis in pneumonia has simplified the statistical approach to this disease. Because of the narrow fatality ranges involved, the generally accepted practice of alternating cases and analyzing

them by type, bacteremia, leukopenia, lobe involvement, duration of disease, therapy, etc., requires unusually large numbers of patients in order to prove the significance of any particular factor. The division into sputum groups, however, permits an unlimited range of fatalities depending only upon the number of cases studied. Such a classification could be duplicated by various investigators and in all probability would not be influenced by seasonal, yearly, or community variations in the virulence of the disease, nor would the usual separation, according to type, bacteremia, leukocyte count, or duration of the disease, be required in order to obtain statistically significant results. Since the primary objective of all studies is to reduce the fatality rate in pneumonia, the division into sputum groups would permit an accurate evaluation of therapeutic agents with greater safety and with relatively few cases. All new drugs submitted for clinical trial would first receive preliminary study in selected patients with initial sputum counts of 20 or less. The efficacy of the drugs could be evaluated in these patients by repeated examination of sputum to determine whether or not they were capable of reducing the number of pneumococci as rapidly as sulfathiazole or sulfadiazine. If the sputum count failed to decrease, or actually increased within a specified time, therapeutic agents of proven value could be readily substituted without subjecting the patients to unnecessary hazards (5, 7). The most promising drugs could then be studied in cases with more than 30 pneumococci per field in the hope that they might effect a significant reduction of the high fatality rate which still obtains with sulfathiazole and sulfadiazine in this group.

#### SUMMARY

The average number of pneumococci per oil immersion field in Wright-stained smears of rusty sputum was determined at 12-hour intervals in 678 cases of pneumococcic pneumonia, exclusive of type III. The close relationship between the highest sputum count during the course of the disease, and the outcome, was shown by the fact that the fatality rate was 2 per cent when the pneumococci did not exceed 10 per field, 9 per cent when 11 to 30 were present, 30 per cent when 31 to 75 organisms were found, and 77 per cent when the number exceeded 75. The fatality

rate was uniformly low in patients with less than 30 pneumococci per field and uniformly high in those with sputum counts above 30, regardless of the type of pneumococcus, duration of the disease prior to therapy, leukocyte count, and blood culture. The unfavorable prognosis usually attributed to type, bacteremia, leukopenia, and late pneumonia, was due chiefly to the higher incidence of sputum counts over 30 per field in such cases. On the other hand, a comparison of cases whose sputum counts fell within the same range showed that age and extent of consolidation had a definite effect upon the outcome since the fatality rate was significantly higher in patients over 54 or in those with consolidation of 2 or more lobes on admission to the hospital. It was therefore concluded that the number of pneumococci in the sputum had the greatest influence on prognosis and that the most important accessory factors were the extent of involvement and the age of the patient. It was also found that modern chemotherapeutic agents did not appreciably reduce the fatality rate in cases with sputum counts below 30, but were distinctly superior to sulfanilamide and serum in cases with counts above 30. The combination of serum and drugs appeared to be no more effective in the latter group than chemotherapy alone.

The authors are deeply indebted to Dr. A. B. Mitchell for his assistance and advice in the preparation and analysis of the tables.

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# TYPE III PNEUMONIA: THE PROGNOSTIC SIGNIFICANCE OF RETICULATION IN RELATION TO THE NUMBER OF PNEUMOCOCCI IN THE SPUTUM, THERAPY, BACTEREMIA, LEUKOCYTE COUNT, AGE, AND DEGREE OF INVOLVEMENT<sup>1</sup>

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The microscopic examination of sputum from patients with pneumonia has revealed that type III infections are distinctly different from those due to other pneumococci. The outcome of type III pneumonia was correlated more closely with the presence or absence of a fibrin-like reticulum in Wright-stained smears of sputum than with the number of organisms per oil immersion field (1, 2). The reticulation appeared to originate from the capsules of the interdispersed pneumococci and was subsequently identified as capsular polysaccharide. A quantitative relationship was demonstrated between the degree of reticulation and the amount of SSS in the sputum and lungs (3, 4). The reticulated type III sputums contained an average of 1,360 mgm. per cent of SSS; whereas, the non-reticulated specimens yielded an average of only 45 mgm. per cent. The amount of SSS in the sputums of the total group of type III cases exceeded by 170 times the amount recovered from types I, II, VII, and VIII cases. Autopsy studies revealed that there was 60 times more capsular polysaccharide in the lungs of type

III cases than in those due to the above mentioned types. The following report is based upon 114 roentgenographically proved cases of type III pneumonia which were studied by the method described in the companion publication on page 207 of this journal. The results are analyzed according to the presence or absence of reticulation in the sputum,<sup>2</sup> as well as the number of pneumococci per oil immersion field. These findings are compared as to prognostic significance with the time-honored criteria, such as bacteremia, leukocyte count, age of the patient, and extent of consolidation.

## RESULTS

The total fatality rate of 33 per cent for type III pneumonia was significantly higher than the average of 13 per cent for all other types. When the cases were grouped according to the number of pneumococci in the sputum (Table I), it was found that the fatality in group A (10 or less per field) was 11 per cent for type III, as compared

<sup>1</sup> Supported by a grant from the Commonwealth Fund to the Michigan Department of Health Laboratories.

<sup>2</sup> Four patients who were non-reticulated on admission to the hospital were finally classed in the reticulated group as the disease progressed.

TABLE I  
The relation of reticulation to sputum counts

Sputum group	Non-reticulated				Reticulated				Total				Other types		
	Cases		Deaths		Cases		Deaths		Cases		Deaths		Cases		Deaths
	Number	Per cent of total	Number	Per cent fatality	Number	Per cent of total	Number	Per cent fatality	Number	Per cent of total	Number	Per cent fatality	Number	Per cent of total	Per cent fatality
A	48	67	2	4	5	12	4	80	53	46	6	11	320	47	2
B	21	29	2	10	18	43	15	83	39	34	17	44	210	31	9
C	3	4	1	33	14	33	10	72	17	15	11	65	101	15	30
D	0	0	0		5	12	4	80	5	5	4	80	47	7	77
Totals	72	63	5	7	42	37	33	79	114	100	38	33	678	100	13

TABLE II

*The relation of therapy to reticulation*

Therapy	Non-reticulated			Reticulated		
	Cases		Deaths	Cases		Deaths
	Num- ber	Num- ber	Per cent fatality	Num- ber	Num- ber	Per cent fatality
Supportive.....	9	1		6	6	
Serum.....	6	0		6	6	
Sulfanilamide.....	4	0		2	2	
Drug and serum*....	2	0		1	1	
Totals.....	21	1	5	15	15	100
Sulfapyridine.....	18	2		5	4	
Sulfathiazole.....	33	2		13	6	
Sulfadiazine.....	0	0		1	1	
Drug and serum*....	0	0		8	7	
Totals	51	4	8	27	18	67

\* The term drug and serum refers to serum plus sulfanilamide in contrast to serum plus sulfapyridine, sulfathiazole, or sulfadiazine.

with the rate of 2 per cent for all other types. The corresponding figures for group B (11 to 30 per field) were 44 per cent and 9 per cent; those for group C (31 to 75) were 65 per cent and 30 per cent, respectively. A further classification of these cases into those with and without reticulation served to clarify this discrepancy. As shown in Table I, the fatality rates in the non-reticulated cases of 4 per cent for group A, 10 per cent for group B, and 33 per cent for group C, were comparable with the averages for all other types. The reticulated cases, however, showed a uniformly high fatality, approximating 80 per cent, irrespective of the number of pneumococci in the sputum. The independent effect of reticulation on the outcome supports the concept that the ability of the type III pneumococcus to produce

capsular polysaccharide is highly significant in prognosis. That the number of pneumococci in the sputum also plays a role is shown by two observations: (a) the fatality rate in the non-reticulated cases was proportional to the number of pneumococci in the sputum, and (b) the incidence of reticulation in the total group of type III cases was 25 per cent when the sputum count was less than 30, as compared with 86 per cent when it exceeded 30 per field. Because reticulation was a more accurate index of the severity of type III pneumonia, it has replaced sputum counts in the remaining tables as a basis for comparison with other prognostic factors.

The analysis of the outcome in reference to therapy is presented in Table II. The fatality rate in the non-reticulated cases was as low with serum, sulfanilamide, and even with supportive treatment, as it was with modern chemotherapeutic agents. The superiority of sulfapyridine and sulfathiazole became evident in the reticulated cases where the fatality was lowered from 100 to 67 per cent. This significant reduction in fatality does not entirely account for the well known beneficial results with sulfapyridine and sulfathiazole in type III pneumonia. The effectiveness of these drugs is also due to their ability to decrease the number of pneumococci in the sputum of non-reticulated cases, thereby preventing the formation of excessive amounts of capsular polysaccharide as the disease progresses. To date, not a single non-reticulated case of type III pneumonia has become reticulated while under treatment with sulfapyridine or sulfathiazole, whereas four such patients developed reticulation during the administration of large doses of serum and subsequently expired.

TABLE III

*The relation of bacteremia to reticulation*

Sputum group	Negative blood culture				Positive blood culture				Totals		
	Cases		Deaths		Cases		Deaths		Cases		Deaths
	Num- ber	Per cent of total	Num- ber	Per cent fatality	Num- ber	Per cent of total	Num- ber	Per cent fatality	Num- ber	Per cent of total	Per cent fatality
Non-reticulated.....	67	80	4	6	1	5	0	0	68	64	6
Reticulated.....	17	20	11	65	21	95	18	86	38	36	76
Totals.....	84	79	15	18	22	21	18	82	106	100	31

The low bacteremic incidence of 21 per cent associated with a high fatality rate of 82 per cent in bacteremic type III pneumonia is representative of other reports in the literature. In Table III, the results are analyzed with reference to reticulation and to blood culture. Reticulation was present in 95 per cent of the 22 bacteremics, including all 18 who died. Among the 84 non-bacteremic cases, only 20 per cent were reticulated, but these included 11 of the 15 deaths. Thus, the reticulation which was almost invariably present in bacteremic type III cases may largely account for their unusual severity, since the fatality rate was also high in reticulated cases without blood stream invasion.

The role of leukopenia in the outcome of type III pneumonia is shown in Table IV. The fatality rate was 18 per cent in the cases with leukocyte counts over 10,000, 32 per cent in those with white counts between 5,000 and 10,000, and 77 per cent in those with less than 5,000 cells per cubic millimeter. A study of the table, however, shows that the fatality rate was uniformly low in the absence of reticulation and uniformly high in its presence, regardless of leukocyte count. The higher fatality rate in leukopenia than in leukocytosis is due to the fact that 92 per cent of the former, and only 26 per cent of the latter, had reticulated sputum. It is thus apparent that the outcome of type III pneumonia is correlated more closely with the presence or absence of reticulation than with the leukocyte count.

The data are analyzed according to age in Table V. In the non-reticulated group, 4 of the 5 deaths occurred in patients over 54 years of age. Cardiac failure was an accessory factor in the death of all 5 cases. While the fatality rate in the reticulated cases was not influenced by age, it should be noted that the incidence of reticulation increased from 0 to 50 per cent with advancing years.

The fatality rate in cases with consolidation confined to one lobe was 29 per cent as compared with 44 per cent in those with involvement of two or more lobes (Table VI). When these cases were broken down according to the presence or absence of reticulation, no significant differences were found which could be attributed to the degree of involvement. The higher fatality

TABLE IV  
*Relation of leukocyte count to reticulation*

Sputum group	10,000 and over			5000 to 9999			Less than 5000			Unknown			Totals		
	Cases		Deaths	Cases		Deaths	Cases		Deaths	Cases		Deaths	Cases		Deaths
	Num-ber	Per cent of total	Num-ber	Per cent fatality	Num-ber	Per cent fatality	Num-ber	Per cent of total	Per cent fatality	Num-ber	Per cent of total	Per cent fatality	Num-ber	Per cent of total	Per cent fatality
Non-reticulated	57	74	3	5	13	68	1	8	0	1	1	1	72	63	7
Reticulated....	17	26	11	65	6	32	12	92	83	7	7	7	42	37	79
Totals,....	77		14	18	19		13		77	8		8	114	100	33



TABLE V  
*Relation of age to reticulation*

Sputum group	5 to 29 years						30 to 54 years						55 years and over						Totals		
	Cases			Deaths			Cases			Deaths			Cases			Deaths			Cases		
	Num-ber	Per cent of total	Num-ber	Per cent fatality	Num-ber	Per cent fatality	Num-ber	Per cent of total	Num-ber	Per cent fatality	Num-ber	Per cent fatality	Num-ber	Per cent of total	Num-ber	Per cent fatality	Num-ber	Per cent fatality	Num-ber	Per cent of total	Per cent fatality
Non-reticulated.....	16	100	0	0	37	62	1	3	19	83	4	21	50	33	38	45	72	63	7		
Reticulated.....	0		0		23	38	19				14	74	50		19		42	37	79		
Totals.....	16	14	0	0	60	53	20	33			17	45	33		114	100			33		

among the cases with consolidation of more than one lobe could be explained by the fact that a distinctly higher percentage showed reticulation. It would, therefore, appear that the degree of involvement in type III pneumonia does not play as significant a role in determining fatality as it does for other types.

DISCUSSION

The results in type III pneumonia differed from those obtained for all other types in two important respects. The outcome was not influenced by the degree of involvement and depended more upon the presence or absence of reticulation than upon the number of pneumococci in the sputum. The unfavorable prognosis customarily attached to the presence of bacteremia and leukopenia was attributable to the fact that reticulation was almost invariably associated with blood stream invasion or low leukocyte count. Age was the only other factor which significantly affected the outcome in type III cases. The above data, coupled with the additional evidence already presented (1 to 4), make it difficult to escape the conclusion that the prognosis in type III pneumonia is primarily influenced by the amount of SSS produced by this organism as it grows in the lungs. Such a concept may be readily correlated with other well known facts concerning the type III pneumococcus. This organism, because of the mucoid character of its growth, yields the largest amount of capsular polysaccharide under appropriate cultural conditions (5). It is also common knowledge that sputum from type III patients frequently requires dilution before exhibiting a positive Neufeld reaction. The disease induced by this pneumococcus is characterized by a predilection for the aged, a prolonged clinical course, a lobular distribution, and a relatively low bacteremic incidence, associated with a high fatality rate despite massive doses of serum (6 to 9). At necropsy, the consolidation has a characteristic gelatinous appearance and multiple lung abscesses are often demonstrable. In the above respects, type III more closely resembles Friedländer's pneumonia than infections due to the other common types of pneumococci (9, 10). The similarity between Friedländer's and type III pneumonia is further emphasized by the fact that in both diseases the

## PROGNOSTIC DATA IN TYPE III PNEUMONIA

TABLE VI

*Relation of involvement to reticulation*

Sputum group	One or less lobes				More than one lobe				Totals		
	Cases		Deaths		Cases		Deaths		Cases		Deaths
	Num- ber	Per cent of total	Num- ber	Per cent fatality	Num- ber	Per cent of total	Num- ber	Per cent fatality	Num- ber	Per cent of total	Per cent fatality
Non-reticulated.....	56	70	3	5	16	47	2	13	72	63	7
Reticulated.....	24	30	20	83	18	53	13	72	42	37	79
Totals.....	80	70	23	29	34	30	15	44	114	100	33

high fatality rates are largely attributable to the extraordinary capacity of these organisms to form excessive amounts of capsular polysaccharide (2 to 4). The preponderance of the evidence would seem to justify the segregation of type III cases into a separate and distinct group, which requires special therapeutic, clinical, and laboratory studies.

We have not been impressed by the value of serum, either alone or coupled with sulfonamide drugs, in type III pneumonia. Several of our patients went into circulatory collapse shortly after serum was given, which suggested that harm rather than benefit had resulted from its administration. The futility of giving antibody to these patients is also obvious from the quantitative analysis for SSS, where it was shown that from 100 to 500 million units would be necessary even to neutralize the amount of polysaccharide in the lungs (3). Even in the non-reticulated cases, the sputum may become reticulated despite the administration of large doses of serum (2). For these reasons, we believe that serum should be discarded in the treatment of uncomplicated type III pneumonia except in rare instances where the pneumococci may have acquired resistance to the sulfonamide drugs.

The problem of specific treatment of type III pneumonia thus resolves itself into the use of chemotherapeutic agents of proven value. When reticulation was absent on admission to the hospital, its development was prevented by sulapyridine and sulfathiazole. The most prompt effects in early reticulated sputum were obtained from the use of sodium sulfathiazole, in large doses, intravenously. The number of pneumococci decreased within 24 hours and the re-

ticulation disappeared within 36 hours after the institution of therapy. The clinical response in reticulated cases, however, was rarely dramatic and failure to obtain improvement following a drug effect in the sputum was suggestive of complications, particularly lung abscess. The reticulated type III cases are still a therapeutic challenge and, together with group C and D cases due to other types of pneumococci, are suitable for the final evaluation of new agents that have proved effective in experimental infections and in the milder human pneumonias.

## SUMMARY

The type III pneumococcus differs from all other pneumococci in its capacity to produce excessive amounts of capsular polysaccharide, as manifested by a fibrin-like reticulum in Wright-stained smears of rusty sputum. In the present study of 114 cases of type III pneumonia, reticulation in the sputum was by far the most important factor in determining the outcome. Thus, the fatality rate was 79 per cent when reticulation was present and only 7 per cent when it was absent. Furthermore, the fatality in reticulated cases was uniformly high, regardless of the number of pneumococci in the sputum, the blood culture, the leukocyte count, and the extent of the consolidation, but was partially influenced by the factor of age. The unfavorable prognostic significance customarily attached to bacteremia and leukopenia in type III pneumonia was attributable to the fact that reticulation was almost invariably present in such cases. The outcome in the non-reticulated cases, as in the other types, was dependent upon the number of pneumococci

ble a simultaneous recording and direct comparison of electroencephalograms and acoustic respirograms (Figs. 1 and 2).

This recording system has two outstanding advantages. The necessary amplification is carried out at high frequencies, where vacuum tube amplifiers are simple in construction and stable in operation. Secondly, the only connection between the subject and the apparatus is a small contact microphone. This makes it possible to obtain records without the subject's knowledge that his respiration is being studied, and under conditions which provide a minimum of restriction to his movements, and none to the moving current of air.

By the graphic representation of the sound which is produced by the movement of air currents in the upper respiratory tract, the respiratory cycle can be analyzed into several clearly recognizable phases.

At the onset of inspiration, there is a sudden rapid up-curve as the moving column of air gathers velocity and as the sound gathers volume. At some point on this curve, there is a levelling off to form something approximating a plateau,

which in turn may be level, may continue slowly to rise, or may gradually fall. At the end of inspiration, there is a sharp drop back to the base line. Then comes a quiet lull during the interphase between inspiration and expiration, a phase which is lost to view in mechanical methods of registering the respiratory movements of the thorax or abdomen. This interphase terminates where the onset of expiration causes a second rapid rise in the curve, a second plateau as the expelled column of air attains a more or less constant velocity, and again a sharp drop at the end of expiration. Thus, there is an up-slope, an approximate plateau, and a down-slope for both the inspiratory and the expiratory phases of respiration, and two interphases, one between inspiration and expiration, and another between expiration and inspiration. The sharpness of these slopes, the inclinations of the plateaux, the relative durations of the various phases and interphases, and their smoothness or jerkiness, all give insight into variations in the respiratory function. Not only are gross irregularities of rhythm clearly revealed, but also many subtler patterns which elude ordinary inspection and such methods of

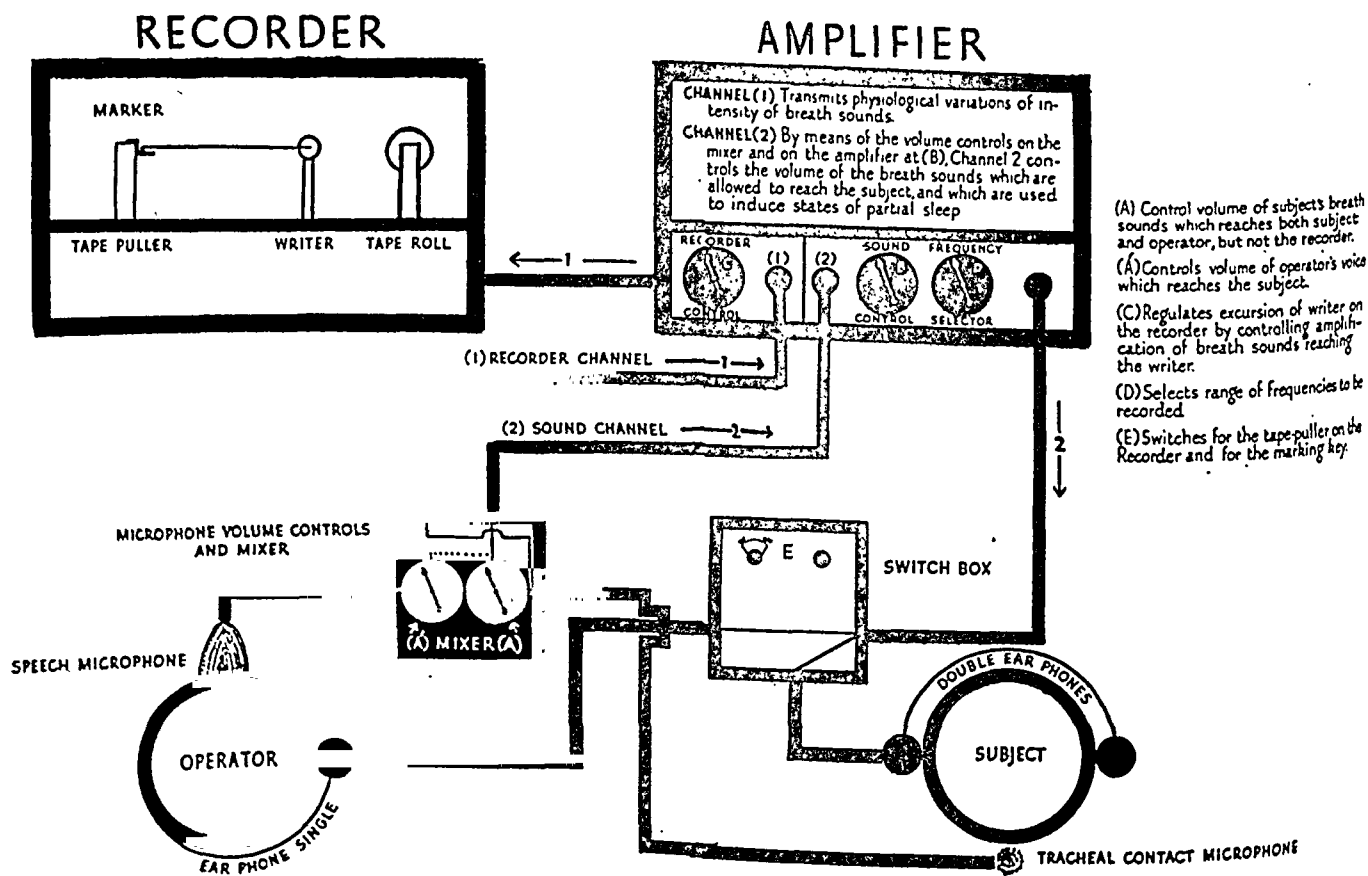


FIG. 1. ACOUSTIC RESPIROGRAPH

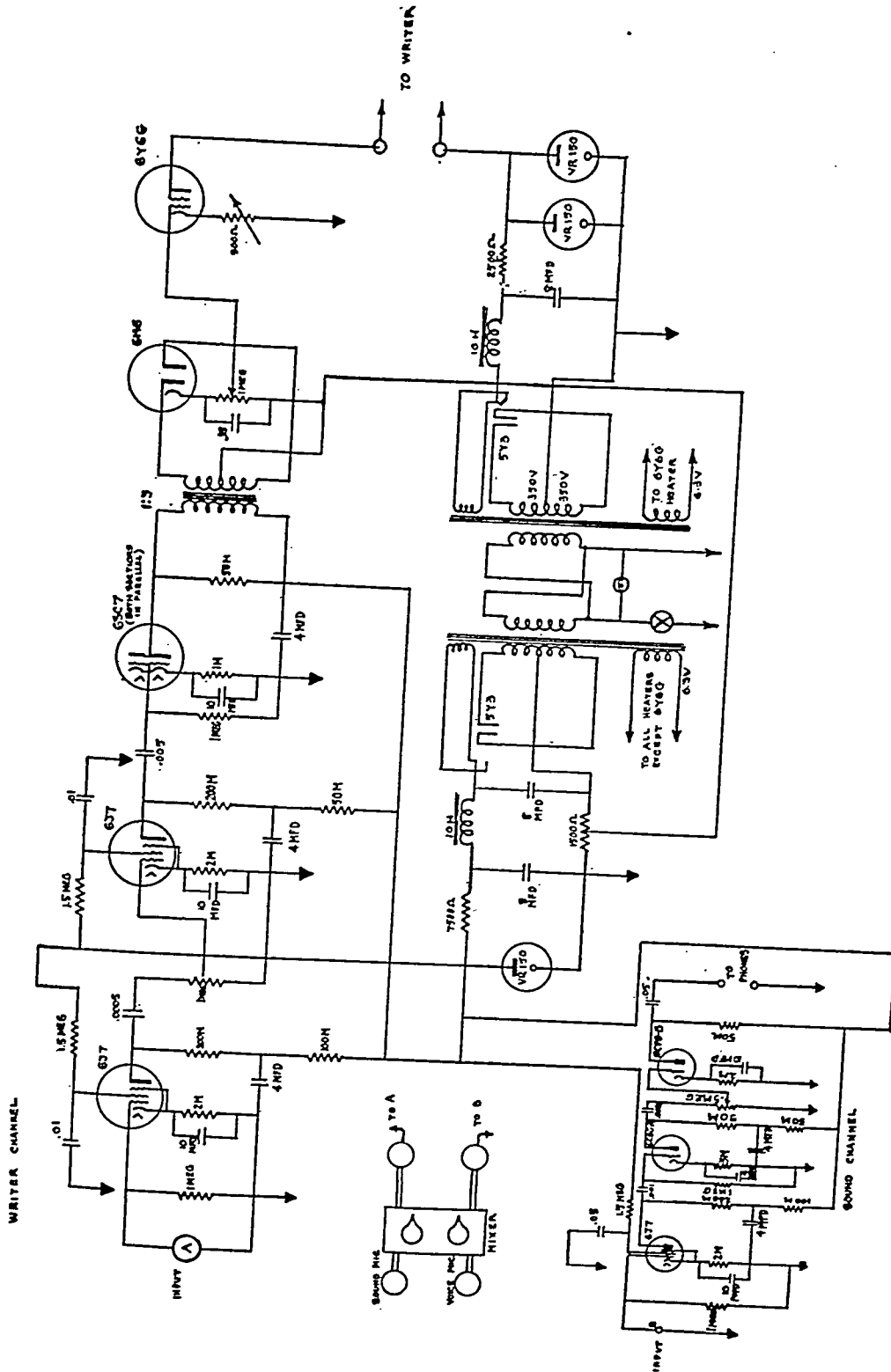


FIG. 2. CIRCUITS FOR ACOUSTIC RESPIROGRAPH

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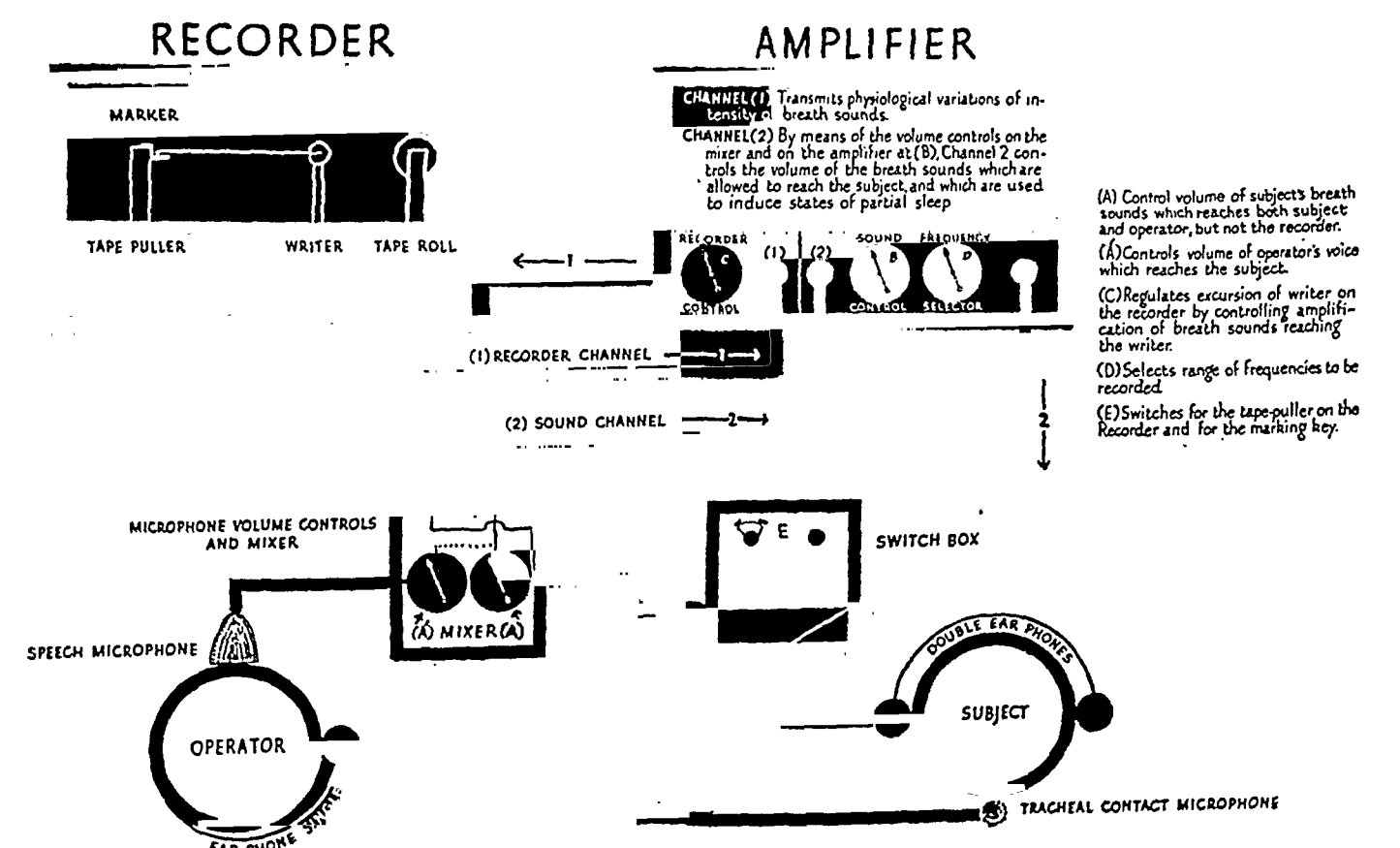
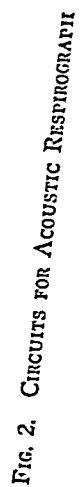


FIG. 1. ACOUSTIC RESPIROGRAPH



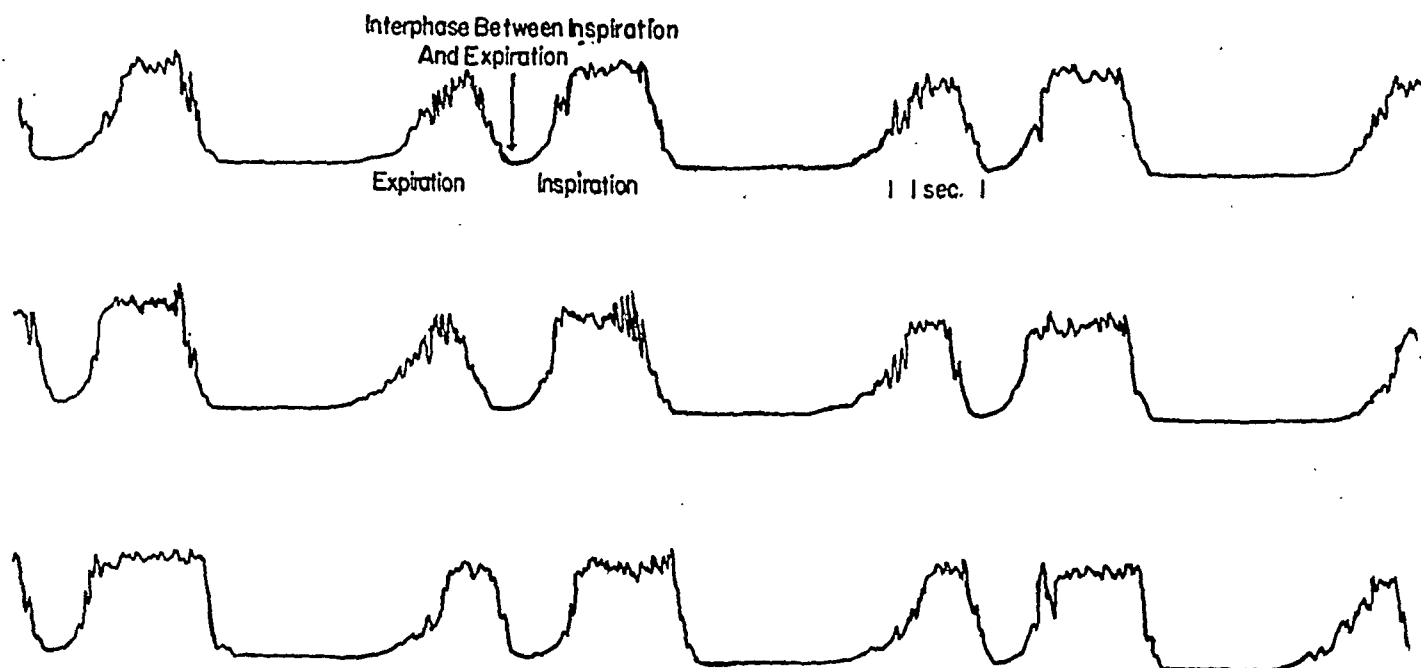


FIG. 3. ACOUSTIC RESPIROGRAM (Read from right to left)

recording as spirometry, pneumography, and air-ometry. Larval Cheyne-Stokes patterns, periods of relative apnoea, curious types of cogwheel respiration, and marked prolongation of one or the other interphase have been observed. They appear to have significant qualitative and quantitative correlations with the process of falling asleep, with the state of sleep itself, with states of anxiety or of undifferentiated emotional tension, etc. Detailed studies of these physiological and psychological correlations will be reported later (Figure 3).

Furthermore, since the whole moving current of respired air probably contributes to the acoustic energy of the breath sounds, the height of any point on the curve must be related in the first instance to the velocity of a given volume of air passing under the microphone at that instant. It follows, therefore, that theoretically the area subtended by the curve and the base-line must represent one integral of the volume of moving air. For this reason, quantitative volumetric correlations between the acoustic respirogram and the minute respiratory volume may be expected. In Figure 3, however, it is evident that the area subtended by the curve which represents inspiration is not equal to that subtended by the curve which

represents expiration. Preliminary experiments with an artificial larynx-trachea-lung have shown that this is because the acoustic energy is influenced also by the direction of motion of the respired air in the respiratory tract. Therefore, separate calibrations are necessary for expiration and inspiration, unless a position can be found for the microphone where a summation occurs of all resonances and of all accompanying acoustic energies. A study aimed to demonstrate quantitative volumetric relations is now under way.

The instrument was developed jointly by one of us (S. M.) and Mr. Paul Traugott of Electro-Physical Laboratories, New York.

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# INTUBATION STUDIES OF THE HUMAN SMALL INTESTINE. XXI. A METHOD FOR MEASURING INTRA-LUMINAL PRESSURES AND ITS APPLICATION TO THE DIGESTIVE TRACT<sup>1</sup>

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## INTRODUCTION

The measurement of the gradient of intra-luminal pressure within the digestive tract is of fundamental physiological importance because, in any hollow tube, fluid will flow from an area of high pressure to an area of low pressure, and we cannot fully understand the flow till the pressures are known. The clinical importance of intra-luminal pressure measurements in hollow viscera is well exemplified by the syndromes of hyper- and hypotension in the cardiovascular system. These disorders were recognized only after the development of an instrument of precision for their detection. This report deals with the development of such an instrument, suitable for use within hollow viscera, and with the results of its application to the digestive tract.

While numerous individual observations on the intra-gastric pressure, and occasionally on the intra-intestinal pressure, have been made with varying degrees of accuracy, it was not till Brody, Werle, Meschan, and Quigley (1) devised an optical recording system in 1940 that an attempt to obtain pressure gradients on animals was successful. Our attempt has been to do the same on man.

The requirements of an acceptable method are: (1) the pressure-sensitive elements must be small enough to be swallowed by the patient without discomfort and to be passed readily to any desired location, as checked fluoroscopically; (2) the instrument must be simple to calibrate in terms of absolute pressure, and capable of responding to small changes, of the order of 5 mm. of water; (3) it must provide simultaneous records of the

pressure at several locations in the tract; (4) the determinations, if not made continuously, must be sufficiently frequent to record all significant fluctuations; (5) the pressure-sensitive elements must measure the average hydrostatic pressure in their vicinity, and must not be subject to error caused by contact with the gut wall or small pieces of solid contents; (6) the device must not cause either obstruction or mechanical stimulation. The instrument to be described is capable of meeting all these requirements.

## PRINCIPLE OF THE METHOD

The principle of the method depends on the closure of an electrical contact by a flexible diaphragm when the pressures on the two sides of the diaphragm are made equal. We have mounted the diaphragm in the wall of a hollow cylindrical brass capsule which is attached to a length of rubber tube. Through the tube, fine insulated wires are led to the contacts, one of which is stationary, the other being carried on the flexible diaphragm but so adjusted that the two contacts just touch when both sides of the diaphragm are exposed to equal pressures. When the capsule and tube have been swallowed, a known pressure applied to the inside of the capsule by way of the tube is varied until the electrical contact is just closed by the diaphragm. The value of this pressure is then equal to that in the lumen of the gut in the vicinity of the capsule. It is clear that any number of similar capsules may be attached to the same rubber tube at intervals along its length. The pressure in the gut at each of these capsules is then determined separately, by reference to the appropriate electrical circuit, and the determinations are repeated as often as may be necessary.

This procedure may be made entirely automatic.

<sup>1</sup> Aided by a grant from the Smith, Kline and French Laboratories.



The known pressure is caused to vary in a cyclic manner by means of a motor-driven reciprocating pump over a range from less than atmospheric to some value considerably in excess of the greatest pressure expected in the intestine. Evidently at two points in its cycle of variation, the known pressure will be equal to the unknown, and the equality of the two can be indicated by either the opening or the closing of the contacts. The pump is arranged to carry a pen in synchronism with the piston back and forth above a strip of record paper. This pen is actuated by an electromagnet which, through suitable intervening circuits, operates at each closing or opening of the capsule contacts, thus printing a dot on the paper when the known pressure is equal to the unknown. The position of the dot printed on the paper is thus directly related to the pressure in the gut in the vicinity of the capsule. The paper is advanced by a feed mechanism and the series of dots form a semi-continuous record of the unknown pressure. When several capsules are to be used, their respective pens are filled with different colored inks so that the individual tracings may be readily identified even though they overlap on the record (Figure 1).

The rate at which the pump operates is determined by the desired time resolution and would ultimately be limited by the velocity of the cyclic pressure wave in the connecting tube and by lag in the operation of the pens. (The latter limitation could be removed by recording photographically, or with a stylus on electrolytically-active paper of the "Teledeltos" type.)

Although the cyclicly varying pressure has been referred to above as "known," it is quite unnecessary to know its actual value, since direct calibration is easy, and indeed preferable, because it takes into account any errors due to "set" of the diaphragms and lag of the pens. It is accomplished simply by placing the capsules and their connecting tube in a closed pressure bottle connected to a water manometer.

#### CONSTRUCTION OF THE INSTRUMENT

We have designed and built an experimental model of the instrument in which three capsules and pens record once every two seconds. On the basis of prior work with inflated balloons, it was thought that this rate would be great enough to resolve all significant fluctuations. Unfortunately, this proved not to be the case, and it is

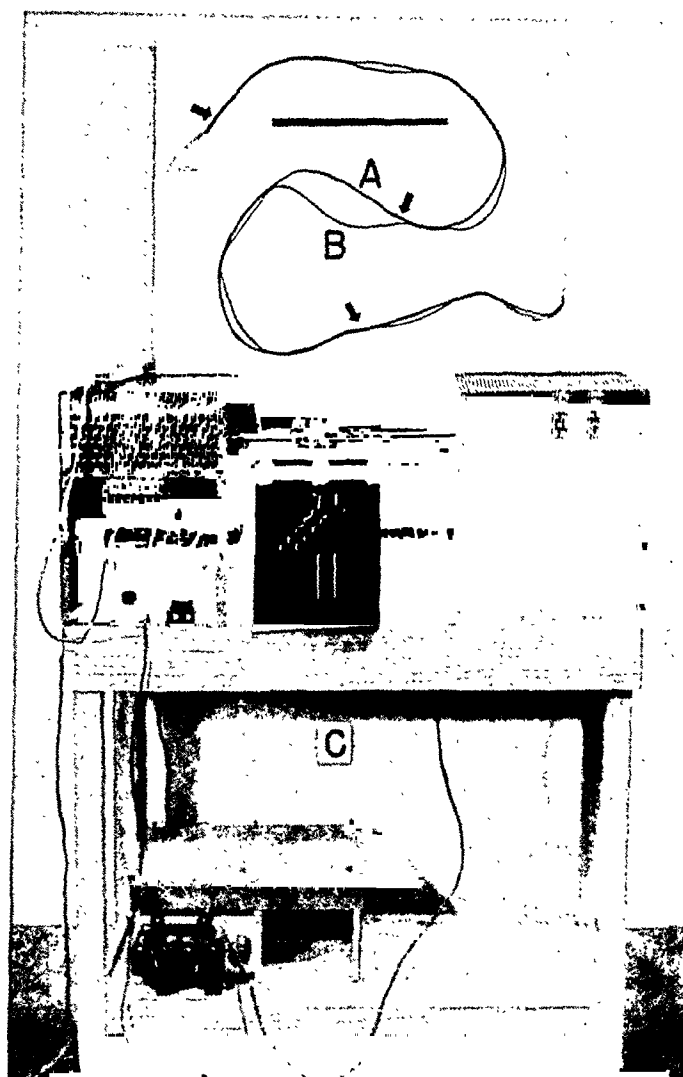


FIG. 1. PRESSURE RECORDING APPARATUS

(A) Tube having three pressure-sensitive capsules whose positions are indicated by the arrows. (B) Auxiliary tube with balloon, to carry first tube down the intestine. (C) Assembly of motor-driven pump, writing pens, and amplifier.

our present opinion that the rate should be about four times as fast, that is, once every half second. The record paper moves about a third of a millimeter per second, so that consecutive dots are displaced on the time axis by about two thirds of a millimeter. This spacing is adequate and should be retained in subsequent models. While ideally it would be possible to have the instrument record the unknown pressure twice during each cycle, by using both the opening and closing of the contacts in the capsule, the lag in the magnetically operated pens, which amounted to about 1 per cent of the cycle duration, slightly displaced the dots printed on the downstroke from those printed on the upstroke. This difficulty can be avoided, but the added complication was not deemed warranted, and the machine was arranged to print only on the closure of the capsule contacts.

The pressure capsules are hollow brass cylinders, approximately 5 mm. in diameter and 26 mm. in length. The longitudinal section is shown in Figure 2. The

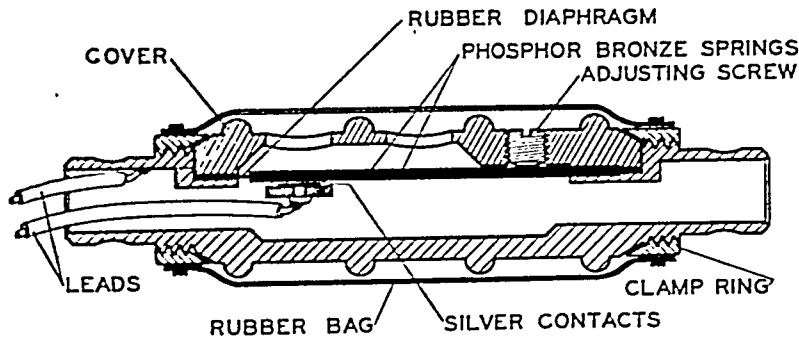


FIG. 2. SCALE DRAWING OF PRESSURE-SENSITIVE CAPSULE  
Overall length 26 mm.; diameter approximately 5 mm.

central part is milled out to form a flat seat for the diaphragm cover which is cut from thin rubber. The diaphragm is held in place by a mating cover secured by the two clamp rings. It is reinforced by two phosphor-bronze strips 0.005 inch thick by 0.093 inch wide. One of these strips is soldered at one end to the body of the capsule and carries at its other end the moving contact. The other strip is soldered at one end to the cover, and is acted on by the adjusting screw, in order that the contacts may be set just to touch each other when both sides of the diaphragm are exposed to the same pressure. The stationary contact is mounted in a small bakelite strut which is cemented in the bore of the capsule body. It is of course necessary to make sure that there is a free passage through the bore for the cyclic pressure wave as well as for the fine wires from other capsules. Actually two wires from each capsule to the recording instrument are not needed, as the capsule bodies are all connected to the grounded side of the circuit. The external slack bag is also made of thin rubber, and serves to keep intestinal contents from entering the perforations in the cover and mechanically interfering with the operation of the diaphragm. The corrugated surface of the brass parts is necessary to prevent the bag from sealing the holes in the cover, thus causing false pressure indications.

The electrical system needed for indicating closure of the contacts might be very simple in the event that non-automatic measurements were to be made. It might consist merely of a high-resistance voltmeter in series with a dry cell, with the addition of a selector switch if several capsules were to be used. However, in the automatic recording device, an electromagnet must be actuated, and the contacts in the capsule cannot themselves control the magnet current, because, first, the pen must bear against the moving record paper for only a brief period of time whose length should be independent of the duration of closure of the capsule contacts; second, the current necessary to actuate the magnet would cause arcing at the contacts with subsequent erratic operation; third, the transient voltages occurring at the interruption of the magnet current would introduce some hazard to the patient.

For these reasons, suitable vacuum-tube circuits are

employed between each capsule contact and its respective pen magnet. Briefly, these consist of a high- $\mu$  triode, such as the type 6SF5, coupled to a pentode, such as the type 6F6, through a parallel resonant circuit, blocking condenser, and grid resistor; the anode circuit of the pentode includes the pen magnet, and the effective output resistance of the circuit is increased by negative feedback methods to improve the speed of the pen. Normally, both tubes draw current from the supply, but when the contact in the capsule closes, due to the equalizing of the pressures on opposite sides of the diaphragm, the grid of the triode becomes sufficiently negative to cut off the plate current. As a consequence, there is a large transient voltage developed across the parallel resonant circuit. This voltage is in a direction to produce a large increase in the plate current of the pentode, causing the magnet to draw the pen towards the paper. The constants are so chosen that the resonant circuit is somewhat underdamped, and the duration of the transient is long enough to give adequate acceleration to the pen; but due to the underdamping, the magnet current actually falls below the quiescent value before returning to normal, thus giving a decelerating effect just before the pen hits the paper.

As the voltage necessary to over-bias the high- $\mu$  triode is only five or six, and as the impedance of the input circuit can be made as high as half a megohm, the contacts in each capsule have to control a current of only about ten microamperes, so that no arcing or erosion can occur. Furthermore, potentials of the order of five volts introduce no hazard to the patient, even in the event of extensive failure of insulation in the capsules. The pump, recorder, and low side of the electrical circuit are connected to ground as a safeguard against insulation failure in either the pump motor or the rectifier transformer supplying the vacuum tubes.

The pens are brass tubes about 0.5 mm. inside diameter and 1 cm. long, soldered into tubular ink reservoirs 2 mm. in diameter and 1.5 cm. long. In each pen there is a central brass wire whose height in the bore can be adjusted to regulate the delivery of ink. The magnetic system consists of the frame, coil, armature, and spring of a standard Dunn "midget" relay. The pen is attached to the armature by a spring hinge and moves axially in a loose guide on the coil frame. The assembly of pen and

magnet is mounted on a bar which travels with, and parallel to, the piston of the pump.

The pump consists of a brass cylinder with a leather cup-washer piston, driven by a motor with built-in reduction gears. The length of stroke (8 inches) was chosen to give the desired length of scale on the record, since pens and piston move the same distance. The ratio of displacement volume to total clearance volume is determined by the range of pressure to be measured. The average pressure in the system is maintained equal to atmospheric by a capillary leak.

The paper feed mechanism is driven from the pump shaft through a worm gear and chain-sprocket. To hasten drying of the ink a tubular electric heater irradiates the entire width of the paper.

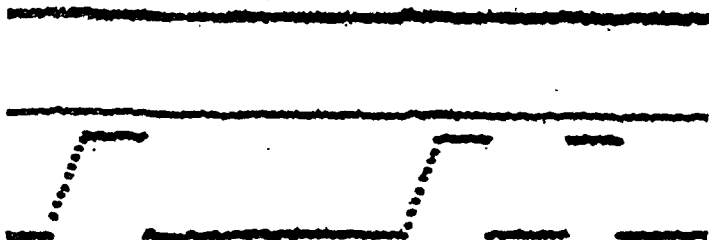


FIG. 3. SAMPLE RECORD IN WHICH THE PRESSURE WAS ALTERED ON ONE CAPSULE ONLY

This shows that the response of the other units is entirely independent of changes applied to the first.

In Figure 3 are tracings recorded with three pressure-sensitive capsules in operation. At the beginning of the record, all three capsules were in air. One of them (lower tracing) was then slowly immersed in water to a depth of 14 cm., and after several seconds removed suddenly. The third time it was immersed suddenly to the same depth, and then removed. This record shows the constancy of the curves traced under unvarying pressure, and the reliability with which changes in pressure are recorded. It also shows the independence of the recording channels. A typical calibration record is shown in Figure 4. All three of the capsules were placed in a pressure bottle and covered by 2 cm. of water. The pressure in the bottle was then adjusted to the levels indicated on the record, with the help of a water manometer, and short tracings were recorded at each level. The reliability of the base line (atmospheric pressure + 2 cm. of water) before and after the calibration is to be noted. The calibration curves obtained from this record for each recording channel are shown in Figure 5. Because of the small range of pressures, they are nearly linear; theoretically, they are small portions of hyperbolas.

#### RESULTS

Fourteen observations have been carried out on nine subjects. The total recording time for the group was 28½ hours with either one, two, or three capsules in action simultaneously. Of the

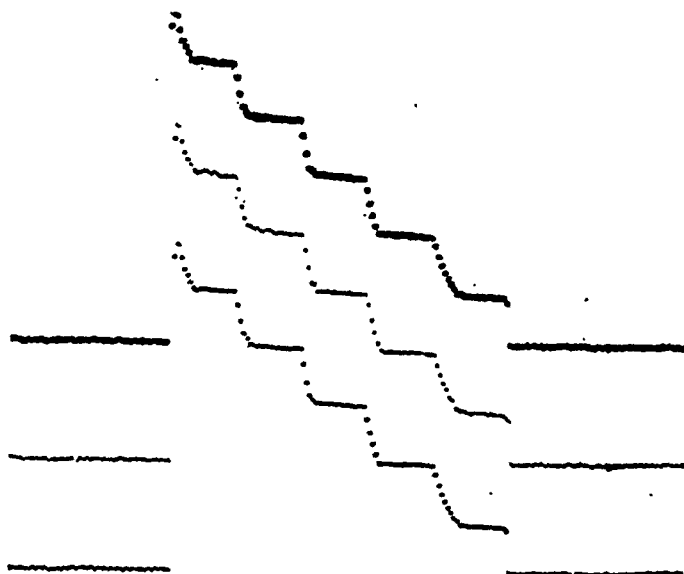


FIG. 4. CALIBRATION RECORD

Time reads from left to right. All three capsules in pressure bottle are under 2 cm.  $H_2O$ . Pressure was suddenly increased to a high value. By a controllable leak on the bottle, the pressure was then allowed to subside to 50 cm.  $H_2O$ , where it was held for about half a minute, and then dropped successively to 40, 30, 20, and 10 cm.  $H_2O$  to give a step-like calibration. Note that the base-line to which the trace returns is the original 2 cm.  $H_2O$ .

nine subjects, four were normal individuals whose good health was substantiated by a history, a complete physical examination, a gastric analysis, and a gastro-intestinal fluoroscopy. Each was used twice. The remaining five subjects were patients

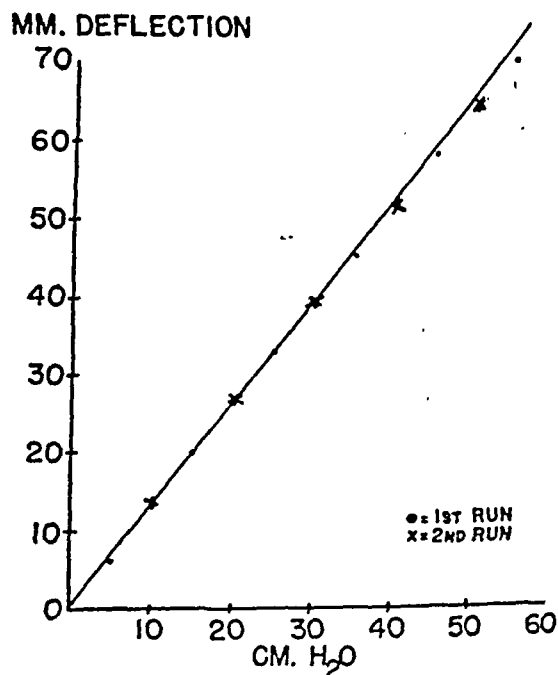


FIG. 5. THE CALIBRATION CURVE

with either no digestive disorders or a diagnosis of a functional indigestion, arrived at by exclusion after a thorough study in the hospital. Each experiment was conducted with the subject lying upon the fluoroscopic table for frequent observation.

From these records typical periods were selected for detailed analysis. Figure 6 shows a coincident jejunal pressure record and a balloon record. The intra-luminal pressures (A) show characteristics typical of all our records, *viz.* (1) a basal pressure of 8 to 10 cm. of water, and (2) phasic rises in pressure, in this case to 30 cm. H<sub>2</sub>O or less. In contrast, the balloon record which in a rough way is a measure of the capacity of the gut lumen over a limited area shows a pattern of far greater variability indicating that not every contraction or relaxation of the intestinal musculature brings about an appreciable change in intra-luminal pressure.

Figure 7 shows an attempt to place the pressure regulating mechanism of the gut under stress. A single instrument was placed in the mid-jejunum and water was given by mouth. During the next 15 minutes, a basal pressure averaging 11.2 cm. of H<sub>2</sub>O was maintained. A succession of phasic pressure waves, gradually increasing in intensity till levels of 35 to 55 cm. of H<sub>2</sub>O were reached,

now appeared. The true mean pressure during this period was 13 cm. H<sub>2</sub>O.

A balloon just distal to the recorder was now blown up to form an obstruction to flow. Water was injected proximal to the balloon until the pressures obtained represented those occurring above an acute artificial intestinal obstruction. Waves of pressure, equalling in their intensity those of the unobstructed gut but more prolonged in their duration, now appeared. From the 25th to the 40th minute of the experiment, the average basal pressure was 15.7 cm. H<sub>2</sub>O and the mean pressure 20.3 cm. H<sub>2</sub>O.

To obliterate the waves and raise the tonus of the intestine, morphine sulphate, gr.  $\frac{1}{6}$ , was given subcutaneously at 59 minutes. Only one phasic wave appeared thereafter but a rise in the basal pressure to 17.8 cm. H<sub>2</sub>O was observed during the subsequent 10 minutes.

Figure 8 represents the relation of gastric fundus, gastric antrum, duodenal cap, and distal duodenal pressures. Records from three points were simultaneously obtained. Tracings A and B show essential identity, save for slight gravity (altitude) differences in the pressures in the two ends of the stomach. The subject had taken two glasses of a thin water-barium mixture to drink. Pressure waves affected the antrum and

### SIMULTANEOUS INTESTINAL PRESSURE & BALLOON RECORDS

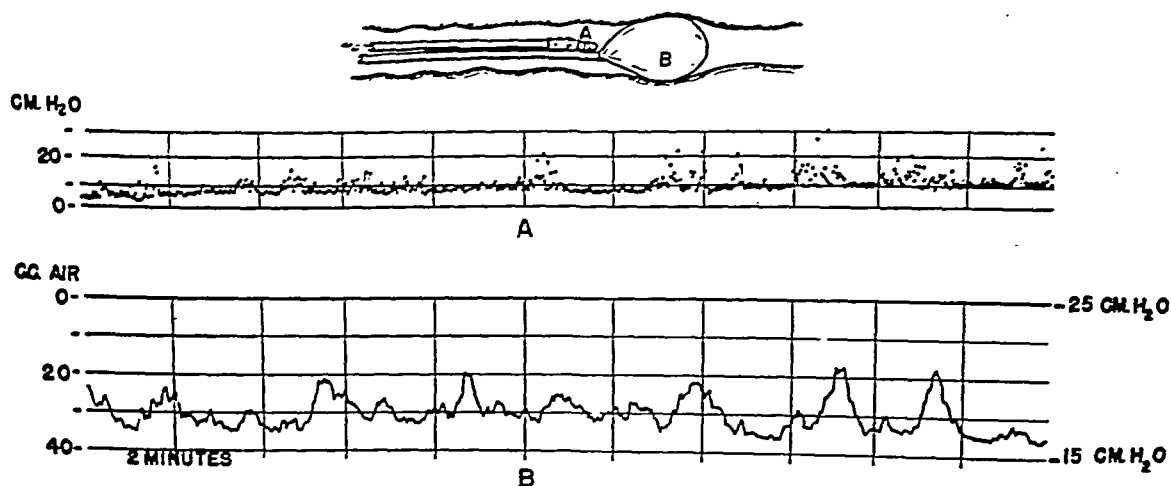


FIG. 6. COMPARISON OF A BALLOON RECORD AND A PRESSURE RECORD

(A) The intra-luminal pressures and the location of the instrument. (B) The balloon tracing. It is obvious that the two do not coincide.

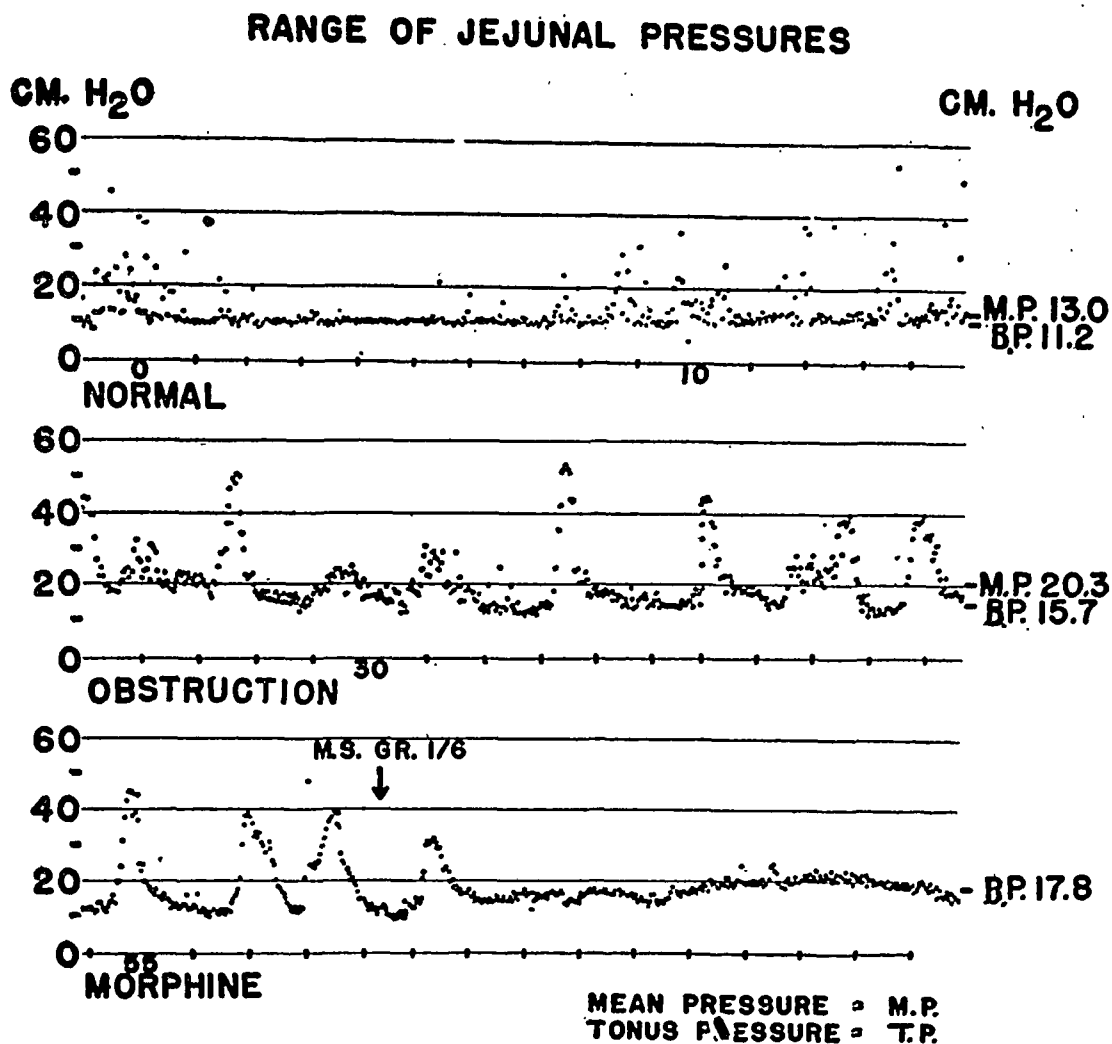


FIG. 7. PRESSURES RESULTING ABOVE AN ARTIFICIAL MECHANICAL OBSTRUCTION OF THE JEJUNUM

the fundus alike and apparently had nothing to do with the gastric peristalsis visible by fluoroscopy. While three antral peristaltic waves per minute might reach the pylorus, these general gastric contractions might occur only once in one and a half or two minutes. Nevertheless, the appearance of high pressures in the duodenal cap coincided roughly with the peaks of intra-gastric pressure. Fluoroscopic observation of the stomach suggested that if a wave of antral peristalsis relaxed the pylorus at some time close to the height of the gastric contraction, then gastric contents entered the duodenal cap and the cap pressure rose sharply. Thus the rise in cap pressure bore an approximate rather than an exact relationship to the peak of gastric pressure and more nearly corresponded in time to the opening and closing of the pylorus, provided this occurred while the intra-gastric pressure was high enough to force the gastric contents into the duodenum.

That such was not always the case, however, is suggested by Figure 8, showing the result of moving the recorder along the intestine from positions A, B, and C to positions B, C, and D. Gastric pressure now maintained an average level slightly above the duodenal, and gastric evacuation occurred as the pylorus opened, without slowly recurring rises in intra-gastric pressure being noticeable.

Figure 9 shows the simultaneous pressure changes at three widely separated points in the upper small intestine of a patient with a kidney stone, whose "reflex" gastro-intestinal disturbances, chiefly belching, nausea, distention, and abdominal cramps, led to a tentative diagnosis of intestinal obstruction by the admitting officer. In the mid-duodenum, the basal pressure and mean pressure were respectively 11.8 cm. H<sub>2</sub>O and 14.8 cm. H<sub>2</sub>O. In the upper jejunum, these pressures were 13.8 cm. H<sub>2</sub>O and 15.2 cm. H<sub>2</sub>O, respectively,

and in the mid-jejunum, they were still higher, basal pressure 14.6 cm.  $H_2O$  and mean pressure 16.1 cm.  $H_2O$ .

#### DISCUSSION

The distribution of intra-luminal pressures in the digestive tract produces the flow of contents and, in all probability, accounts for most digestive symptoms, since stretching the gut is a major cause of intestinal pain. Before we can begin to

define the normal limits of gastro-intestinal pressures, however, a great deal more work must be done. Hidden sources of error especially must be discovered and eliminated. In the past, for instance, much work has been invalidated by a failure to differentiate between the pressure produced by the contraction of the bowel wall upon its fluid contents and that produced by the contraction of the wall upon a balloon. Pressures transmitted by a balloon are not only modified by

#### GASTRO-DUODENAL PRESSURE AND FLOW

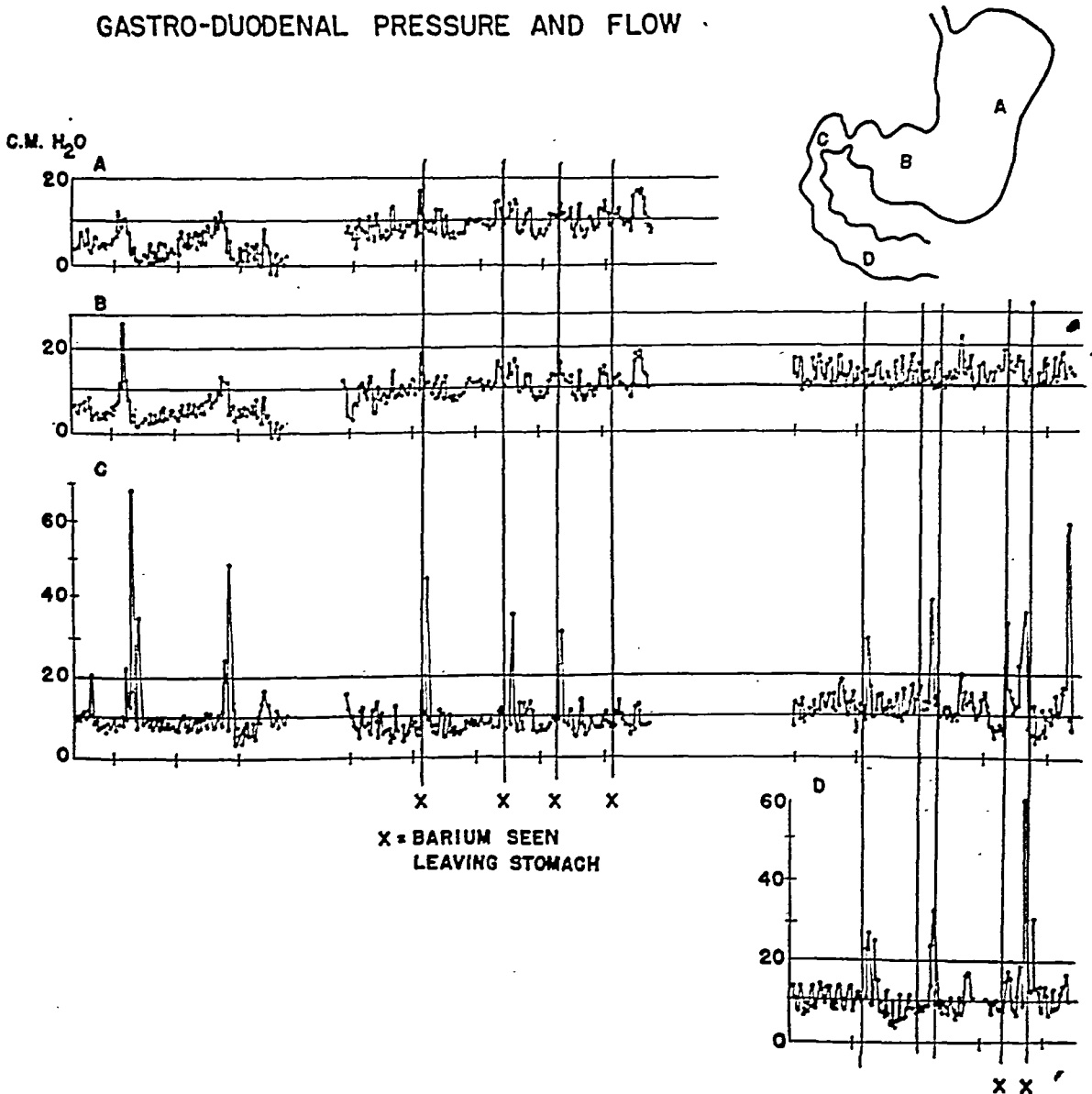


FIG. 8. CORRELATION OF PRESSURES RECORDED AT THREE POINTS IN THE GASTRO-DUODENAL REGION SIMULTANEOUSLY WITH THE FLOW OF BARIUM FROM THE STOMACH TO THE DUODENUM

## SIMULTANEOUS SMALL INTESTINAL PRESSURES

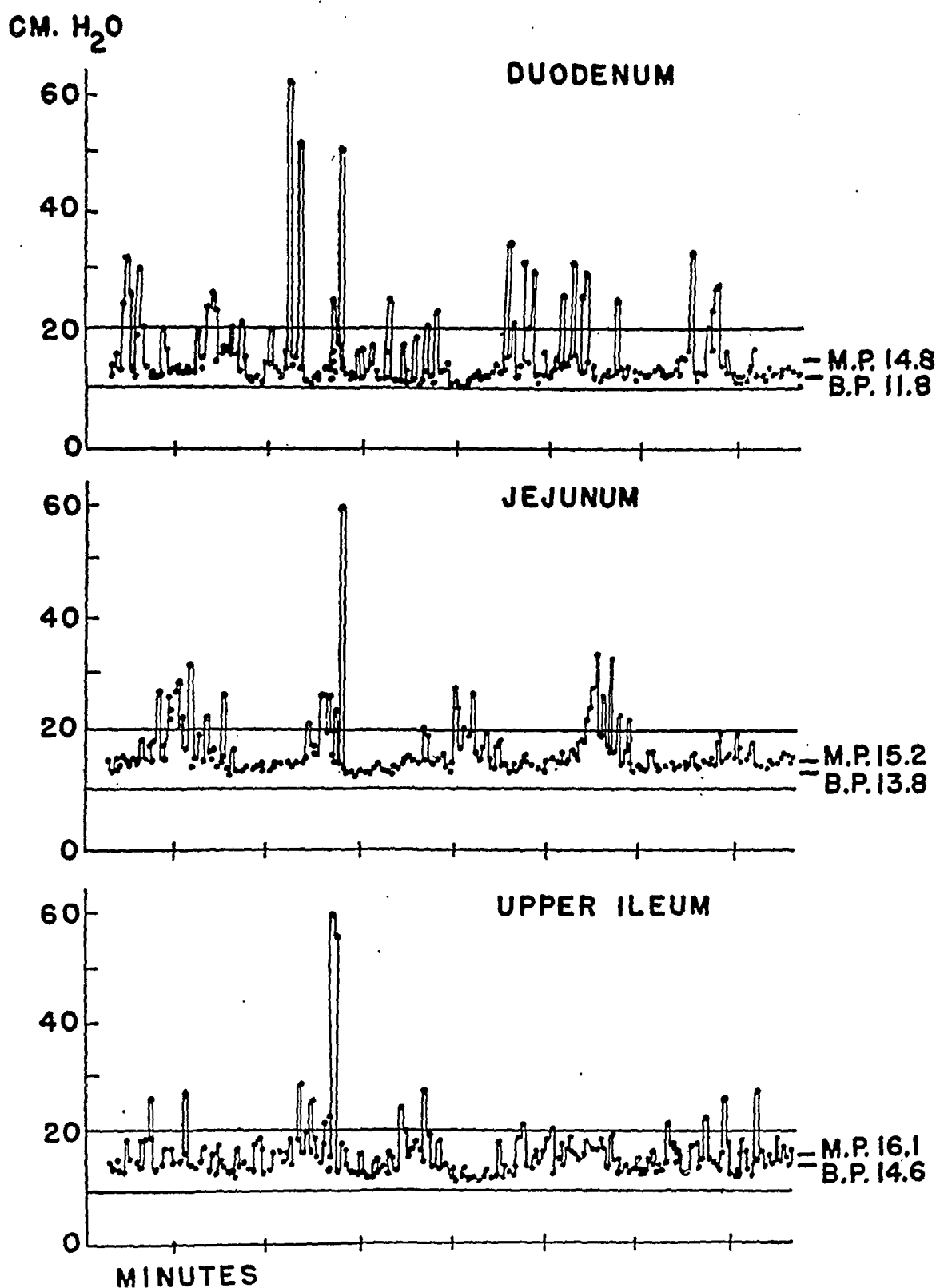


FIG. 9. PRESSURES RECORDED FROM A CASE OF "REFLEX" VOMITING AND DISTENTION

the elasticity of the rubber but "by the inability of the balloon content to escape from the area involved in the contraction. For this reason, pressures recorded by a balloon system may

grossly exaggerate the true intra-luminal pressure developed under normal circumstances which allow the content of a contracting segment to move downstream. It is also obvious that the intra-

luminal pressure is the sum of the pressure of the abdominal wall upon its contents, the pressure of the viscera upon one another, and the intrinsic pressure of the intestinal wall upon its content. The altitude of the recording units must be known, since within the stomach of a standing patient, the pressure will be as much higher in the antrum than in the pylorus as a water column of comparable height. When the subject is supine, with both recorders at the same altitude, the pressure at opposite ends of the stomach may be equal.

We feel that our scattered observations on man are comparable to the studies of Brody, Werle, Meschan, and Quigley *et al.* (2 to 5) on the dog, because vertical and horizontal fluoroscopy convinced us that the sources of error mentioned were eliminated or else equally applicable to all recorders. The same pattern of a basal pressure with superimposed phasic pressures of 30 to 60 cm. occurs in man as in the dog, though the basal pressure in man is somewhat higher. In the mechanism of gastric emptying, some difference exists in that Quigley's dogs appeared to empty their stomach contents into the cap in response to local pressure changes in the pre-pyloric region, though he has not yet shown that the general intra-gastric pressure was not likewise elevated at these times. Our human subjects frequently emptied gastric contents when the total intra-gastric pressure was at or near its peak, rather than in time with pre-pyloric peristaltic contractions, many of which occurred without the expulsion of barium unless this coincided with a rise in general intra-gastric pressure. However, we have not yet been able to record the pressure in the distal antrum at the moment of contraction, as distinct from the general intra-gastric pressure. The low viscosity of our thin water-barium mixture may also play a part in rendering antral contractions incapable of isolating a pre-pyloric chamber. We incline to agree with Alvarez (6), Barclay (7), and Wilson and Irving (8) in thinking that human antral peristalsis does not partition the antrum from the rest of the stomach save under special circumstances, such as just before complete emptying of the stomach takes place.

The pressures above the artificial obstruction showing a sustained level of 10 to 18 cm., with phasic pressures to 40 or 50 cm.  $H_2O$ , exceed

those recorded by Wangensteen (9) of 4 to 14 cm.  $H_2O$ , rising to 20 to 30 cm.  $H_2O$  in a clinical case of mechanical obstruction of more than 48 hours duration. His reading was made by thrusting a needle connected to a manometer into the distended gut at the operating table. Earlier in the patient's course, before distention took place, the intra-luminal pressure may have been higher.

The single record of a rising gradient of internal pressure in the presence of a marked functional disorder proves nothing in so far as Alvarez' theory of intestinal gradients is concerned but is placed on record as an observation of some interest, to be interpreted only when other material of a comparable nature is obtained.

The method is accurate, free from the disturbances of pulse and respiration, and records at a frequency that is probably adequate for most purposes; but the clinical authors have found it rather like an old Swiss watch—it takes the original makers to repair it when it breaks down. The ideal instrument must be more simple.

#### SUMMARY

1. An instrument for recording in absolute terms the intra-luminal pressures simultaneously at multiple selected points in the human digestive tract, without obstructing the lumen, stimulating the gut locally, necessitating a fasting state, or inordinately disturbing a sick patient, has been designed.

2. Basic pressures of 8 to 10 cm.  $H_2O$  are present in the human small intestine.

3. Phasic pressures rising to 30 or 40 or occasionally even to 50 cm.  $H_2O$  occur spontaneously.

4. Placing the gut under stress, by creating an obstruction or by giving morphine, changes the extremes of intra-luminal pressures by only a few cm. of  $H_2O$  but alters the pressure pattern.

5. The coincident gastric and duodenal pressures attendant on gastric emptying were observed.

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# QUANTITATIVE RELATIONSHIPS BETWEEN BLOOD AND URINE KETONE LEVELS IN DIABETIC KETOSIS

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In the study of ketosis in diabetic patients, certain problems have gone largely unanswered due to the lack of sufficiently accurate methods for blood ketone determinations. With the development of blood ketone methods accurate at low as well as high blood concentrations, it has been possible to study the problems of (1) the renal threshold for the ketone bodies, and (2) the quantitative relationship between the urinary ketone excretion and the blood ketone level.

The studies to be presented summarize the results obtained on (1) 13 diabetic patients in mild ketosis, produced by withdrawing insulin for 12 to 24 hours, a ketogenic diet for 12 to 24 hours, or a combination of a ketogenic diet and insulin withdrawal; and (2) 7 patients entering the Los Angeles County Hospital in moderate to severe ketosis (several in diabetic "coma"). In both types of patients, after initial levels of blood and urine ketone bodies were obtained, insulin and fluids were given as required by the individual case, and blood and urine ketone levels were followed until ketosis, as indicated by the qualitative urinary acetone test (modified Lange test), disappeared. The blood ketones<sup>1</sup> were determined by the Barnes-Wick (1) method, and the urine ketones by the Van Slyke (2) gravimetric method in most instances.

Table I summarizes the results obtained in the diabetic patients in mild ketosis, and Table II the results in patients in moderate to severe ketosis. Figures 1 and 2 give the clinical course, during therapy, of two of the most completely studied cases, one in mild ketosis, the other in severe ketosis. These are patients "Ha" and "Wi," from Tables I and II, respectively. The figures illustrate how the data listed in the tables were obtained.

<sup>1</sup> Total blood and urine ketones calculated as beta-hydroxybutyric acid (1).

From the analysis of the data given in these tables and figures, certain facts are apparent. In Table I (record of patients with blood ketone levels between 1 and 30 mgm. per cent), it is seen that even with very low blood ketone levels (5 to 10 mgm. per cent) small amounts of the ketone bodies appeared in the urine. This indicates that the absolute renal threshold for the ketone bodies in diabetic patients is very low—under 10 mgm. per cent—in the majority of cases. While it is evident that the threshold for the ketone bodies is very low in diabetic patients, relatively small amounts appeared in the urine, less than 100 mgm. per hour, until blood levels over 20 mgm. per cent are reached. This gives a ratio under 10 for

$$\frac{\text{urine excretion mgm. per hour}}{\text{blood ketones mgms. per cent}}$$

in 10 of the patients listed in Table I. In one patient ("P"), all the ratios were under 10, with one exception, where there was a marked increase in urine output per hour. In two patients ("Ho" and "St"), there were much higher ratios. Certain variations in the ratios at low blood levels can be explained on the basis of marked variations in urinary output per hour.

The great increase in urinary ketone excretion per hour with blood ketone levels over 20 mgm. per cent is seen in Table II (blood ketone levels from 35 to 150 + mgm. per cent). The amount of urine ketones excreted per hour at comparable blood levels varied greatly from patient to patient. As the amount of the urinary ketone excretion is very small as contrasted to the amount utilized (3), this may partially explain this difference. These results in patients in moderate to severe ketosis indicate a complex relationship between the blood ketone level and the urinary ketone output per hour. When the urinary ketone output per hour is charted against the blood level, a

TABLE I  
Ketone excretion in mild ketosis

Patient	Time	Urine volume	Urine ketones*	Urine ketones mgm. per hour Blood ketones mgm. per cent	Blood ketones
	<i>minutes</i>	<i>cc. per hour</i>	<i>mgm. per hour</i>		<i>mgm. per cent</i>
1. "Ha"†	97	414	101	3.4	30
	86	1,150	58	3.5	16.4
	43	1,310	25	1.6	15.5
	52	559	12.9	1.0	12.8
2. "P"†	166	123	141	7.4	19
	192	106	80.5	4.5	14.7 to 21
	55	933	121	22.4	5.4
	40	630	18.3	4.5	4.1
3. "Du"†	201	128	94	5.9	16
	169	41	10.2	5.1	±2
4. "Ho"	269	118	63.7	3.7	21 to 15.8 to 6.3
	53	386	52	17	3.6 to 2.4
	70	283	39	13	2.4 to 3.6
	65	328	42.5	7.9	3.6 to 7.2
5. "Str"	55	71	32.5	5.7	5.7
	60	276	16.4	2.1	7.9
6. "Hal"	315	210	0	0	8 to 1.2
7. "D"†	57	98	14.4	1.12	12.8
	60	110	4.9	0.83	5.8 to 6
	57	69	6.9	2.7	2.9 to 2.2
	66	74	6.3	2.6	1.7 to 3.1
	51	94	6.1	3.2	3.1 to 0.7
8. "Si"	60	38	51	5.8	8.8
	60	138	64	7.9	8.1
	90	504	0	0	4.7 to 2.4
9. "N"	70	107	55.9	6.8	8.2
	60	180	0	0	5.3
	60	162	0	0	2.7
	60	40	0	0	1.4
10. "Q"	175	63	0	0	7.8 to 3.8
11. "B"†	56	115	4.81	0.8	6.0
	62	420	11.8	1.8	6.6
	64	430	35.7	3.6	10.0
	56	573	3.96	1.59	2.5
	43	725	0	0	1.7
12. "Be"	68	±242	±31.5	±6.2	5.1
	180	55	0	0	4.8
13. "St"‡	60	350	14.6	3.4	4.3
	60	370	24	20	1.2
	58	671	13	18.5	0.7
	62	203	7.5	7.5	1.0

\* Calculated for time period listed.

† Urine ketones fractionated into beta-hydroxybutyric acid and acetone. Amount listed represents beta-hydroxybutyric acid.

‡ Urine ketones represent beta-hydroxybutyric acid. (Mercury precipitate dissolved, distilled and reprecipitated.)

hyperbolic type of curve is obtained (Figure 3—patient "St"—Table II). When these data from Figure 3 are expressed as a relationship between blood level and a ratio between urine ketone

excretion mgm. per hour and blood ketone level in mgm. per cent, a straight line is obtained, with the point of origin passing through the blood level around 20 mgm. per cent (Figure 4). In the

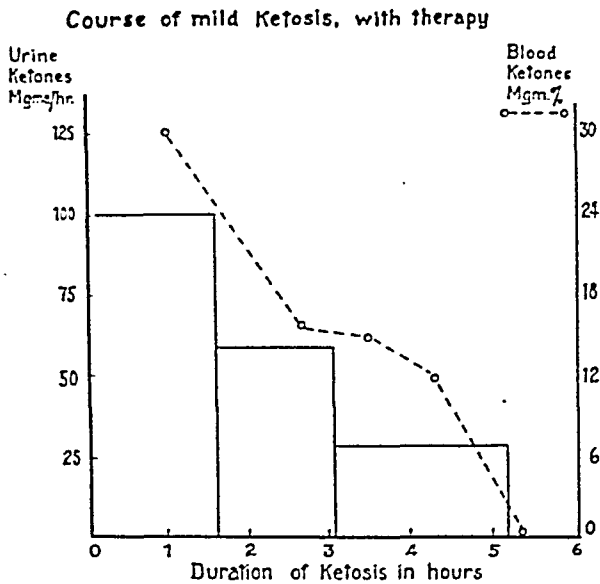


FIG. 1. THE RAPID FALL OF THE BLOOD KETONE LEVEL AND THE SMALL URINARY KETONE OUTPUT AT LOW BLOOD KETONE LEVELS (Patient "Ha," Table I)

majority of the cases, a somewhat similar relationship was found, with a marked falling off of the ratio at blood levels under 20 mgm. per cent.

Studies in 5 patients included the determination of both acetone (including acetoacetic acid and acetone) and beta-hydroxybutyric acid in the urine, at varying blood levels—2 to 78 mgm. per cent. The results are listed in Table III. In patients "Ha" and "K," acetone represented approximately 80 per cent of the ketone bodies in the urine at the lower blood levels. In patient "P," the urinary acetone rose from 11 per cent, at a blood ketone level of 19 mgm. per cent, to 41 per cent, at a blood ketone level of 2.9 mgm. per cent. In patient "B," the urinary acetone rose from 42 to 100 per cent, with corresponding change in blood ketone level from 6 to 1.7 mgm. per cent. In patient "D," there was only a slight increase in percentage of acetone at the lower blood ketone levels.

In studying the data summarized above, certain other points of interest were noted. In all the patients in moderate to severe ketosis, the carbon dioxide combining power was determined repeatedly and synchronously with the blood ketone determinations. The lack of specific correlation in different patients between the carbon dioxide combining power and the blood ketone level is

seen in Table IV. (This reaffirms the previous work of many.) In this table it is seen that one patient with a carbon dioxide combining power of 10 volumes per cent had a blood ketone level of 80 mgm. per cent, while another patient with a carbon dioxide combining power of 16 volumes per cent had a blood ketone level over 150 mgm. per cent. Since the carbon dioxide combining power is only a measure of the alkali reserve, and this is determined by many factors other than the degree of ketosis in uncontrolled diabetes, it is obvious why the two determinations are not more closely related.

Table V shows the low urinary ketone output per hour in a patient with uremia and oliguria. In this patient with blood ketone levels over 150 mgm. per cent, 61.7 to 455 mgm. per hour were excreted in the urine.

#### DISCUSSION

There are few quantitative figures given in the literature on the relationship between blood and urine ketone levels. The impression, however, is given that there is no correlation. As Peters and Van Slyke (4) state: "The relation between blood and urine ketones has not been studied; but the data of Allen, Stillman and Fitz show a peculiar lack of association between the two." In studying the data of these authors (5), it is noted that the total urinary ketone excretion for 24 hours, with varying diets or starvation, is correlated apparently with one or two blood ketone levels. As variations occur during the 24 hours, it is impossible to draw conclusions from their data, although their results do show very small urinary ketone output at low blood ketone levels. Also there are very few references to the renal threshold for the ketone bodies in the literature. The work of Wilder (6) on the injection of beta-hydroxybutyric and acetoacetic acid into dogs, suggested a urinary threshold, since 0.4 gram per kgm. of body weight had to be injected before beta-hydroxybutyric acid appeared in the urine. Briggs and Shaffer (7) conclude from their studies that acetone is a non-threshold substance, and the concentration in the urine parallels the concentration in the blood. These authors, and also Widmark (8), state that under certain conditions acetone can diffuse into the urinary bladder directly from the blood, like alcohol, with-

TABLE II

*Ketone excretion in moderate to severe ketosis*

Patient	Time	Urine volume	Total urine ketones	Urine ketones	Urine ketones mgm. per hour Blood ketones mgm. per cent	Blood ketones
	<i>minutes</i>	<i>cc. per hour</i>	<i>mgm.</i>	<i>mgm. per hour*</i>		<i>mgm. per cent</i>
1. "Wi"	240	589	22,050	5,512	66.5	80 to 86
	250	444	18,120	4,350	58	75
	165	336	9,000	3,270	43.6	75
	260	426	12,560	2,900	39.2	74
	225	613	9,950	2,650	44.2	60
	190	805†	4,630+	1,400+	85.4	16.4
	170	380	1,135	400		
	180	282†	955+	318+		
	170	405	340	120	9.4	12.8
	85	494	0	0	0	1.3 to 2.3
	210	128	136	39	10.4	2.3 to 5.2
2. "St"	290	370	17,305	3,580	70.6	60 to 41.5, av. 50.7
	110	327	2,340	1,270	30.6	41.5
	180	297	1,322	440	16.8	29.9 to 22.9, av. 26
	180	±160(?)	534	±178	±7.7	23.1
	175	284	343	117	6.4	18.3
	125	198	77	37	2.3	16.2
3. "K"‡	240	272	3,014	754	9.7	78
	65	55	56	52	0.8	66
	65	250	116	107	2.1	51.7
	57	137	9	9	0.24	38
	60	200	12	12		
4. "B"	110	292	485	264	7.2	36.9
	130	180	376	174	5.8	29.9
	120	285	237	119	4.9	24.3
	120	365	0	0	0	11.1
	60	110	0	0	0	10
5. "G"	130	128	2,710	1,254	19.6	64
	50	516	922	1,106		
	60	610	397	397	15.9	25
	375	138	311	50	3	
	140	178	40	17	4	4
6. "W"	155	170†	618+	239+	6.6+	36.3
	135	131†	158+	70+	2.7+	26.9 to 25.8
	115	82	98	51	2.2	22.4 to 23.5
	120	121	0	0	0	
	120	21†	0	0	0	5.1
7. "H"§	185	117	977	316		108 to 150+
	135	53	330	147		69

\* Calculated for time period listed.

† Some urine lost.

‡ Urine ketones fractionated into acetone and beta-hydroxybutyric acid. Amount listed represents beta-hydroxybutyric acid.

§ Patient in uremia.

out excretion through the kidneys. Widmark further claims that for acetoacetic acid there is no correlation between the blood and urine curves, indicating secretory activity of the kidney. All this early work, however, is open to the criticism of relatively inaccurate blood methods. Shipley and Long (9) in recent years have established evidence that in the rat, there is a definite renal threshold—between 25 and 30 mgm. per cent.

Our results give definite quantitative data concerning the renal threshold for the ketone bodies, and the correlation between blood level and the urinary excretion per hour. The urinary excretion of ketone bodies varies with the blood level, but does not act similarly to such substances as urea. When the urinary ketone output per hour is charted against the blood level, a somewhat hyperbolic type of curve is obtained, with a

## Course of severe Ketosis, with therapy

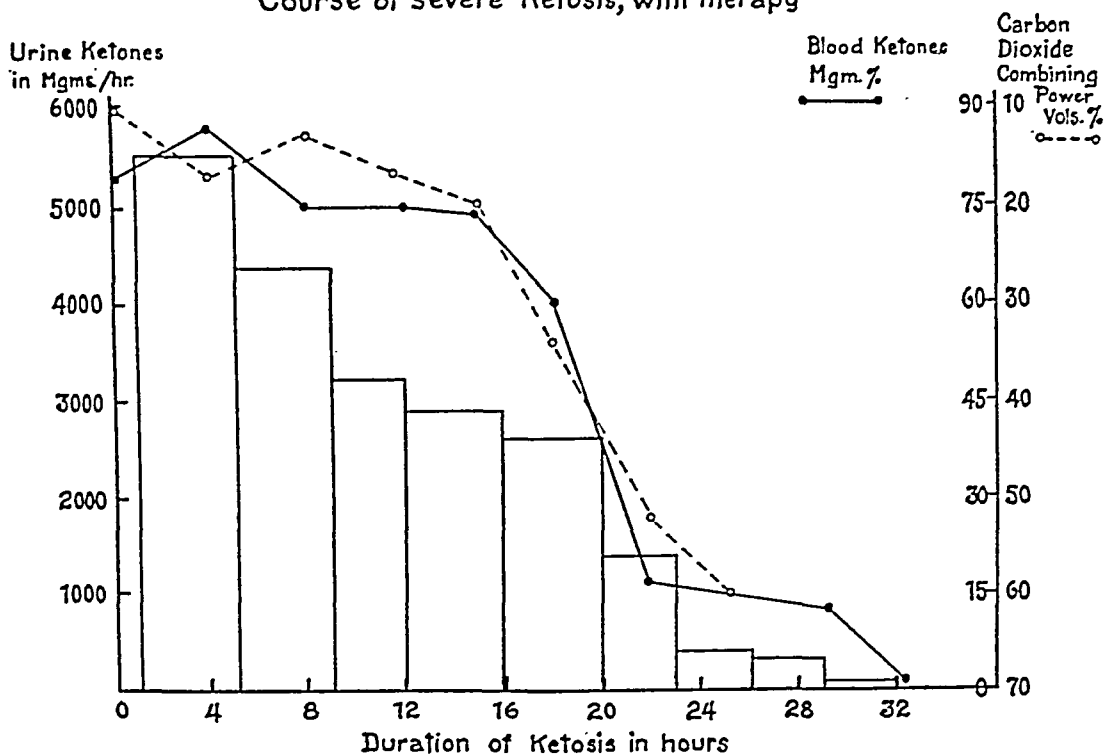


FIG. 2. THE PROLONGED PERIOD OF SEVERE KETOSIS (20 HOURS) WITH THE VERY LARGE URINARY KETONE OUTPUT AT THE HIGH BLOOD KETONE LEVELS (Patient "Wi," Table II)

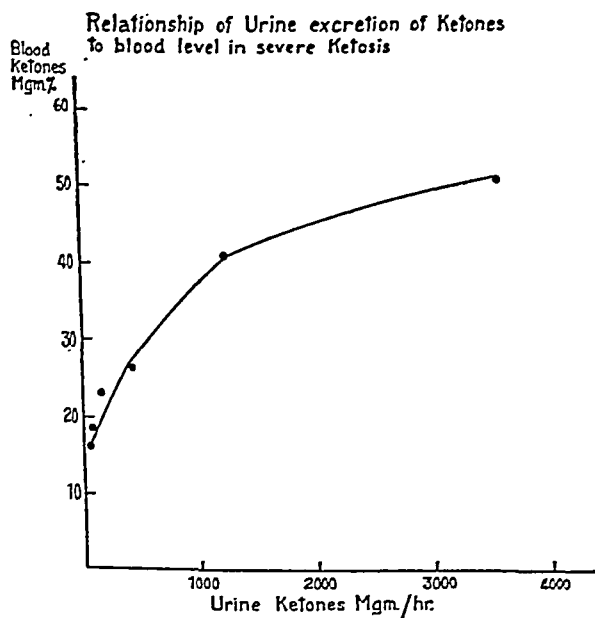


FIG. 3. THE INCREASE IN URINARY KETONE OUTPUT PER HOUR WITH RISING BLOOD KETONE LEVELS (Patient "St," Table II)

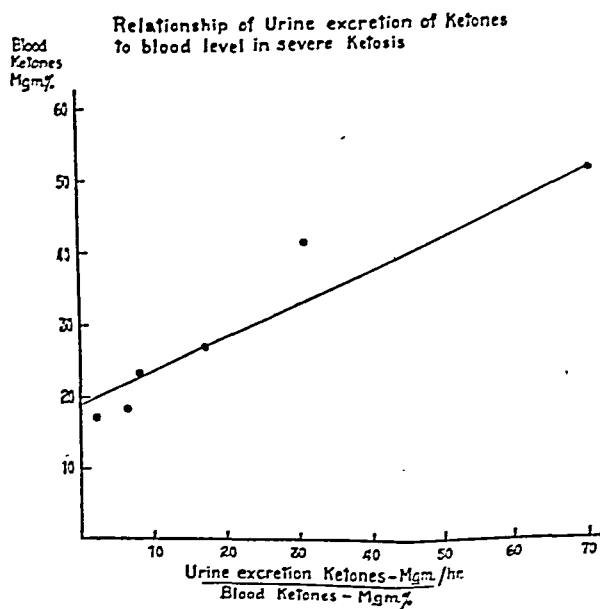


FIG. 4. EXPRESSING THE DATA FROM FIGURE 3 AS A RATIO BETWEEN BLOOD KETONE LEVEL AND THE RATIO BETWEEN URINE KETONES PER HOUR AND BLOOD LEVEL, A STRAIGHT LINE RELATIONSHIP IS OBTAINED (Patient "St," Table II)

TABLE III

*Fractionation of urine ketones into beta-hydroxybutyric acid and acetone\**

Patient	Total acetone in urine	Total beta-hydroxybutyric acid in urine	Acetone	Beta-hydroxybutyric acid	Blood ketones
	mgm.	mgm.	per cent	per cent	mgm. per cent
1. "B"	3.1 2.3 9.6 18 3.4 6.8	4.3 4.5 12.2 38.2 3.7 0	42 33 44 32 48 100	58 67 56 68 52 0	  6 6.6 10 2.5 1.7
2. "K"	34 35 460 84 34 113 38 68	68 385 2,300† 329 56 116 9 12	34 8 17 20 37 50 81 85	66 92 83 80 63 50 19 15	  78 75 78 66 51 38
3. "Ha"	80 84 47 65 41.7 78	163 76 7.1 17.9 17.2 28.9	33 53 87 78 79 73	67 47 13 22 21 27	30   16.4 15.5 12.8 0.5
4. "P"	48.9 46.9 18 9.5 8.4	391 258 85 26.2 12.2	11 15.4 17.4 27 41	89 84.6 82.6 73 59	19 21 to 14.7 5.4 4.1 2.9
5. "D"	23.4 48.1 5.3	270 314 28.8	8 13.3 15.5	92 86.7 84.5	16 2

\* Acetone represents acetoacetic acid and acetone.

† Bladder not completely emptied before, catheter inserted at this point.

TABLE IV

*Lack of correlation between carbon dioxide combining power and blood ketone level*

Blood carbon dioxide combining power	Blood ketones
volumes per cent	mgm. per cent
10	80
13	75
14	64
16	150+
17	86, 78, 30
20	75, 24
23	37
27	75
28	69, 11
34	51, 60, 25
44	10
46	26

marked fall in output with blood levels under 20 to 30 mgm. per cent. (The exact levels vary from patient to patient.) In contrast, when urea excretion per hour is plotted against the blood urea level, a straight line passing through the point of origin is obtained. When the relationship between blood and urine ketones is expressed as a ratio:  $\frac{\text{urinary ketones mgm. per hour}}{\text{blood ketones mgms. per cent}}$  the fact that the ketone bodies are threshold substances is clearly demonstrated, as there is a striking change in the ratio at low and high blood concentrations. This, again, is in distinct contrast to threshold substances, like urea, where the threshold is proportional to the blood concentration. Addis and Drury (10, 11) showed with urea "That under certain special conditions the rate of urea excre-

TABLE V

*Ketone excretion in patient with oliguria and azotemia*

Time	Blood ketones	Urine ketones	Urine volume	Blood sugar	CO <sub>2</sub> C.P.	Fluid intake
	mgm. per cent	mgm. per hour*	cc. per hour	mgm. per cent	volumes per cent†	
9:30 A.M.						1000 cc. N.S. i.v.
10:10						
10:10 to 11:25		?	8	930 (N.P.N. = 80 mgm. per cent)	16	1000 cc. N.S. i.v.
10:40	150+					
11:25 to 12:30		61.7	55			1000 cc. N.S. i.v.
11:45						1000 cc. N.S. i.v.
12:15 P.M.						1000 cc. N.S. subcut.
12:30	150+			740 (chlorides = 335 mgm. per cent)	16	1000 cc. M/6 sodium lactate
12:30 to 2:30		455	150			
1:33	108					
2:30 to 4:45		147	53			
3:42	69			932	28	
4:45						500 cc. blood transfusion
5:55	39					

\* All urines gave a positive qualitative acetone test.

† CO<sub>2</sub> C.P.—carbon dioxide combining power.

‡ Just at death.

N.P.N.—non-protein nitrogen.

N.S.—normal saline.

i.v.—intravenously.

subcut.—subcutaneously.

M/6—sixth molar.

tion becomes directly proportional to the blood urea concentration, so that in any one individual the ratio:  $\frac{\text{urea in 1 hour's urine}}{\text{urea in 100 cc. of blood}}$  is a constant with only narrow limits of variation, over a wide range of blood urea concentrations." The fact that the percentage of acetone (and hence acetoacetic acid) increased proportionally to beta-hydroxybutyric acid at a very low blood level suggests that these substances are non-threshold substances, and would account for the majority of ketone bodies found in the urine under blood levels of 20 mgm. per cent. This is further evidence that beta-hydroxybutyric acid is a threshold substance. It thus appears to belong to the group of threshold substances, and behaves similarly to such electrolytes as chlorides (12), substances with "fixed" thresholds.

These results are of significance clinically. The qualitative urinary acetone test, in the majority of cases, roughly paralleled the quantitative total urinary ketone output. The results in one patient with uremia and oliguria suggest, however, that such a gauge of blood level, *i.e.* urinary acetone, can be used only in the presence of adequate renal function. This has been suggested by others (13, 14), although Briggs (15) claims that it is impossible to have ketone bodies in the blood and not in the urine, due to diffusion directly from the blood to urine in the urinary bladder.

#### SUMMARY

Studies on 20 diabetics in mild and severe ketosis have shown:

1. The low urinary output of ketone bodies (less than 100 mgm. per hour) with blood ketone levels under 20 mgm. per cent. This is probably due largely to the excretion of acetone which appears to be a non-threshold substance.
2. A relationship between blood ketone levels and urinary output:
  - a. Increased urinary output per hour with rising blood ketone levels.
  - b. A renal threshold for beta-hydroxybutyric acid over 20 mgm. per cent.
3. Impaired urinary excretion with renal failure.
4. A lack of correlation between the blood

ketone level and the carbon dioxide combining power.

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# METABOLIC STUDIES IN PATIENTS WITH GASTRO-INTESTINAL CANCER. IV. FAT METABOLISM, A METHOD OF STUDY<sup>1</sup>

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The results of recent studies have added significantly to the understanding of the intermediary metabolism of fat, particularly as concerns the role of the liver in the utilization of fat by the body (1 to 3). On the other hand, the available information concerning the absorption and digestion of fat in the gastro-intestinal tract leaves much to be desired, primarily because of a lack of satisfactory technical methods.

In the course of an investigation of the metabolism of essential dietary factors by patients with gastro-intestinal cancer (4, 5), the ability of these patients to digest and absorb fats was determined. For this purpose, it was necessary to devise a simple and satisfactory quantitative method. A description of this method and the results of its application to patients with various disorders form the subject of the present communication.

## CLINICAL MATERIAL

The clinical material studied consisted of the following:

Two male individuals were admitted to the hospital, one for the surgical treatment of a plantar wart, and the other for a saphenous vein ligation. Both were entirely free of gastro-intestinal complaints and had taken normal diets for several years.

One male patient with gastric cancer was admitted to the hospital for an exploratory laparotomy. At the time of operation, the cancer could not be resected. Both before and after the operative procedure, the patient received an adequate diet which he ate without difficulty. Post-operatively, he remained afebrile and essentially free of all symptoms referable to his disease. There was no significant weight change during the period of observation.

One male patient had had a complete gastrectomy for cancer of the stomach and was not subjected to study until 20 months after the operative procedure. During that time he had lost 35 pounds, but was apparently free from

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any recurrent or metastatic disease. His diet was considered to be adequate and was ingested daily in several small, frequent feedings. His only complaints were postprandial distention and loose, bulky stools.

The one patient with atrophic gastritis was a woman in whom the diagnosis was established by repeated gastroscopic examinations. She had symptoms referable to her disease, but did not suffer from nausea, vomiting, or diarrhea. She had taken a grossly deficient diet for a considerable period.

Two male individuals with hepatic cirrhosis were admitted to the hospital and included in this study. One was a chronic alcoholic who had marked hepatomegaly, moderate splenomegaly, and frequent episodes of icterus, but no ascites, edema, anemia, or diarrhea. The other had hepatomegaly and splenomegaly, with macrocytosis but no anemia. No edema, ascites, jaundice, or diarrhea had been present at any time. The diets of both patients were moderately deficient in animal proteins and vitamin B complex. Both had considerable evidence of hepatic insufficiency as demonstrated by several liver function tests.

## METHODS

### 1. Clinical

a. *Diet and fat load.* From the time of admission, all but 1 of the subjects studied received daily a diet, calculated from standard tables to contain approximately 1.5 grams of protein, 1.5 grams of fat, and 3.0 grams of carbohydrate per kilogram of body weight. The exception, A. Z., was given a diet which by analysis was found to contain 1.1 grams of protein, 0.7 gram of fat, and 4.9 grams of carbohydrate per kilogram of body weight.<sup>4</sup> The patients were allowed a preliminary period of from 3 to 5 days on these diets before any studies were begun, in order that they might become accustomed to the rigid routines employed in their feedings and in the collection of their excreta.

When each subject had taken the basal diet satisfactorily for from 6 to 9 days, a supplement of fat was given in order to obtain a measure of the individual's ability to absorb fat, at both a normal and high level of fat ingestion. This supplement consisted of 2 grams of fat per kilogram of body weight (the "fat load"). Twenty per cent of the fat in this supplement was provided by butter,

<sup>4</sup> The low fat diet in this one instance was employed because it had constituted the basal diet fed to a group of patients who had undergone gastrectomy. The results of that study will be reported in a subsequent communication.

and eighty per cent by heavy cream. The fat load was taken at 7 a.m. of the test day.

The basal diet was eaten in 3 equal meals ordinarily at 7 a.m., 12 noon, and 6 p.m. No food was taken at any other time. However, on the day when the fat load was administered, the meals were taken at 1 p.m., 4 p.m., and 7 p.m.

b. *Collection of feces.* When the subject had taken the basal diet for from 3 to 5 days, a saline enema of 500 ml. was given at 7 a.m. and the returns discarded. From that hour, all stools excreted were collected, for from 3 to 4 days, in a large metal container and kept in a moist ice chest. At the end of each period, another 500 ml. saline enema was given and the returns were added to the stool collection.

From 1 to 4 such collections were obtained from each patient, and the fat content of these collected stools was considered to represent the fecal fat output of the individual under the basal conditions of the study.

At the end of each basal period, each subject was given the supplementary fat meal, and his stools were collected for from 2 to 3 days in 24-hour lots. At the beginning and end of each 24-hour period, the 500 ml. saline enema was administered and the returns added to the proper specimen. Finally, another 2 or 3-day stool collection was obtained in the manner described for the basal period.

## 2. Chemical

*Determination of stool fat.* The Van Slyke combustion method for the determination of lipid in blood (6) was adapted for liquid in stool specimens in the following manner: A homogeneous suspension of the feces in water was made by means of a mechanical stirrer. An aliquot of this aqueous stool suspension, chosen to contain from 0.05 to 0.15 mgm. of lipid carbon, was used. The fat was extracted from this aliquot by 9 volumes of a mixture of equal parts of absolute alcohol and ethyl ether. The extract thus obtained was then dried on a steam bath, cooled to room temperature, and the carbon content of the residue measured.

To ascertain the validity of the results obtained by this adapted technique, some of them were checked by the method of Bloor (7).

## RESULTS

The results of the investigation are presented in 2 parts: 1. The results of studies to test the validity of the method used, and 2. The results obtained when this method was applied to a study of the absorption of fat by the subjects employed.

### 1. Results of studies to test the validity of the method used

a. *Homogeneity of stool suspensions.* Stool collections were diluted with tap water to make a volume of from 500 to 1500 ml., and the whole

stirred mechanically for about 1 hour. To ascertain whether or not this technique provided homogeneous suspensions, immediately after stirring, stearin determinations were made on samples which were removed by a large bore pipette from different levels of suspension. Differences of from 1 to 4.5 per cent were found between the stearin content of the various aliquots (Table I). Thus,

TABLE I  
*Experiments to ascertain the homogeneity of the stool suspension*

Stool of patient	Sample number	Stearin content
		<i>grams per cent</i>
F. P.	1	1.23
	2	1.25
S. L.	1	2.20
	2	2.32
	3	2.20
E. F.	1	1.42
	2	1.43

it would appear that an adequate mixture of the stool specimens was obtained by the technique used.

b. *Comparison of values obtained by the technique used with those obtained by the gravimetric method of Bloor.* It is recognized that the method of Van Slyke (6) measured only the combustible lipid carbon which is extracted by a mixture of equal parts of alcohol and ether from an aqueous suspension. It was desirable, therefore, to ascertain whether or not the stearin values calculated from those of lipid carbon were in agreement with the values for total fat as determined by another (gravimetric) technique. The technique chosen for this comparison was that of Bloor (7).

In Bloor's method (7), the lipid is extracted from 1 volume of aqueous suspension by 9 volumes of a mixture of 1 part ether and 3 parts alcohol. This extraction mixture of Bloor is not the same as that used in the present investigation. Accordingly, it was necessary also to determine whether or not different values were obtained by the use of different extraction mixtures.

Ten ml. samples of each of 2 different suspensions of feces were diluted to 100 ml. with Bloor's solution (alcohol 3 volumes: ether 1 volume) and the fat content of the extract deter-

mined. By the gravimetric method of Bloor, the 10 ml. samples were found to contain 0.145 gram, and 0.117 gram of fat; by the gasometric method of Van Slyke, the fat (stearin) content of the extracts were 0.165 gram and 0.1165 gram, respectively, or 11 and 0 per cent greater than those obtained by the former procedure (Table II).

TABLE II

*Comparison of the fat analyses in stool suspension and in evaporated milk by the adapted gasometric technique of Van Slyke et al., and that of Bloor*

Material	Fat content by method of		Fat content by method of	
	Van Slyke	Bloor	Van Slyke	Bloor
	extracted with alcohol-ether 1 : 1		extracted with alcohol-ether 3 : 1	
Stool suspension number 1, 10 ml. samples.	0.17	0.17	0.165	0.145
Stool suspension number 2, 10 ml. samples.	0.124	0.122	0.116	0.117
Evaporated milk number 1, 10 ml. samples.	1.03	0.97	0.93	0.95
Evaporated milk number 2, 10 ml. samples.	1.04	0.99	0.99	0.93

When the 10 ml. samples of the above 2 stool suspensions were extracted with the mixture used by Van Slyke (alcohol 1 volume: ether 1 volume), the fat contents of the extracts determined by the method of Bloor were 0.170 gram and 0.122 gram, values 17 and 4 per cent greater than those obtained gravimetrically by the use of the Bloor solution. Furthermore, the values obtained by both methods when the Van Slyke solution was used were in better agreement than were those which were obtained by extraction with the Bloor solution. By the gasometric technique and use of the Van Slyke solution, the fat (stearin)<sup>5</sup> contents of the samples studied were 0.170 and 0.124 gram.

Determinations of the same nature also were made of the fat content in two 10 ml. samples of evaporated milk. By the gravimetric and gasometric techniques, the Bloor solution extract con-

tained 0.95, 0.93 and 0.93, 0.99 gram respectively. The values obtained when the Van Slyke solution was employed were, for the gravimetric technique, 0.97 and 0.99 gram, and for the gasometric method, 1.03 and 1.04 grams (Table II).

Thus, it would appear that the values of fat in stool suspensions and in milk, determined by the gasometric technique of Van Slyke, are in good agreement with those obtained by the gravimetric method of Bloor. This agreement is better still when the Van Slyke solution is used in both methods for extraction of the lipid.

## 2. Measurement of the absorption of fat from the gastro-intestinal tract of the individuals studied

a. *Normal individuals.* Two normal male subjects (F. P. and P. R.) were fed the standard diet which contained 1.5 grams of fat per kilo, or a total of 135 and 120 grams per day. During the basal period, neither individual excreted more than 6.3 grams of stearin (fat) per day (Table III). The daily stearin outputs of F. P. for 3 days ranged from 5.0 to 6.3 grams and averaged 5.5 grams, and those of P. R. for 4 days ranged from 3.1 to 4.0 grams, and averaged 3.5 grams. These average excretions of stearin represented, for F. P. and P. R., 4 and 3 per cent respectively of their daily fat consumption.

When the fat supplement was administered to the 2 normal subjects, a sharp increase in the fecal fat output resulted. In the first instance (F. P.), the stearin excretion during the 48-hour period after the ingestion of the fat load rose from the average base level of 5.5 grams per day to 12 grams per day. This increase of 6.5 grams per day represented a loss of 7.6 per cent of the administered fat supplement. Differently expressed, 92 per cent of the fat load had been absorbed. Forty-eight hours after the ingestion of the fat meal, the stearin output of this individual returned to the base level.

In the second instance (P. R.), a significantly increased output of fecal fat was noted only during the 24-hour period which followed the fat load test. In that interval, the subject excreted 12.2 grams of stearin in contrast to his average basal excretion of 3.5 grams per day. This increased fat output represented a loss of 6 per cent of the

<sup>5</sup> Mgm. stearin = mgm. lipid carbon  $\times$  1.15.

TABLE III  
*The excretion of fat (stearin) in the stools of the individuals studied*

Subject	Disease	Excretion of fat while on basal diet		Days	Average fat excreted. Fat ingested	Average amount of fat excreted per day above the basal output after the ingestion of the fat supplement	Days	Extra fat excreted after fat supplement
		Range	Average					
		grams			per cent	grams		per cent
F. P.	Normal	5.0 to 6.3	5.5	3	4.0	6.5	2	7.6
P. R.	Normal	3.1 to 4.0	3.5	4	3.0	8.7	1	6.0
A. A.	Gastric cancer	4.5 to 10.4	6.5	16	7.0	8.5	1	7.0
A. Z.	Gastrectomy for cancer	26.5 to 32.1	29.4	6	73.0	120	1	90.3
M. Z.	Atrophic gastritis	9.6 to 19.5	14.5	6	14.0	29	2	22.0
E. H.	Cirrhosis of liver; chronic alcoholism	7.0 to 9.5	7.6	6	8.1	8.8	1	7.5
E. M.	Cirrhosis of liver	4.9 to 5.0	4.95	3	5.0	2.4	3	3.0

supplement, and indicated that 94 per cent had been absorbed.

In summary, 2 normal adult males, who ingested a constant diet, absorbed 96 and 97 per cent of the fat content of that diet. Furthermore, the fat load of the amount employed does not decrease significantly their ability to absorb fat from their gastro-intestinal tracts.

b. *Patients with gastric disease.* In this group are included 3 individuals: One bearing gastric cancer (A. A.), 1 who 20 months before the present study had undergone a complete gastrectomy for the removal of a gastric cancer (A. Z.), and 1 with atrophic gastritis (M. Z.).

During a basal period of 16 days, the patient with gastric cancer excreted from 4.5 to 10.4 grams of stearin per day, or from 5 to 11 per cent of the dietary fat ingested. The administration of the fat supplement was followed during the next 24 hours by a fecal stearin output of 15.5 grams, or 7 per cent of that load. The fecal excretion of stearin returned to within basal levels (6.7 to 6.8 grams per day) within 48 hours after the fat meal. The findings indicate, therefore, that this patient who bore a gastric cancer apparently absorbed from 89 to 95 per cent of his dietary fat, values only a little lower than those absorbed by the normal individuals. The addition to his diet of a large fat supplement did not decrease this efficiency of absorption.

In sharp contrast to the findings thus far presented were those noted in the patient who had

undergone a total gastrectomy. During his basal period of 6 days, this patient excreted from 26.5 to 32.1 grams of stearin, or from 66 to 80 per cent of that ingested.<sup>6</sup> The one-day addition to his diet of 130 grams of fat as a supplement resulted in an increase of the fecal stearin output during the next 24 hours of 120 grams. This would indicate an absorption of only 10 per cent of the fat load. The return to his basal stearin excretion occurred within 48 hours after the ingestion of the fat supplement.

These observations indicate that not only did the gastrectomized patient have a considerable steatorrhea while taking a comparatively low fat diet, but the addition of the fat load to this diet significantly increased the fat loss.

A detailed investigation into the nature and cause of steatorrhea in the gastrectomized patient is beyond the province of the present study but is presented in a subsequent communication. However, the possibility that a relationship might exist between the absence of a gastric mucosa and the occurrence of steatorrhea was examined in a patient who had marked and generalized atrophic gastritis (M. Z.).

During the 6 day basal period, this patient (M. Z.) excreted from 10 to 20 per cent of the fat ingested. Although these stearin outputs were not as great as were those of the gastrec-

<sup>6</sup> It is to be recalled that the diet of this subject included only 0.7 gram of fat per kilo.

tomized patient (66 to 80 per cent), still they were considerably above the normal values (3 to 4 per cent). Furthermore, the administration of the fat supplement to this patient with atrophic gastritis was promptly followed by an increased stearin excretion of from 28 to 30 grams per day for 48 hours. The increase represented an output of 22 per cent of the fat meal, and was significantly more than those of the normal individuals who excreted 6 and 7.6 per cent of their fat supplements.

Thus, it would appear that the patient who lacked a stomach, and the patient whose gastric mucosa was markedly atrophic, suffered from an impaired ability to absorb fat from their gastro-intestinal tracts. This impairment was accentuated by the ingestion of a fat load. Although no conclusions can be drawn from such studies on isolated patients, it is possible that some relation may exist between the presence of normal gastric mucosa and the ability to absorb fat from the intestinal tract.

c. *Patients with hepatic diseases.* The fact has been demonstrated that patients with hepatic cirrhosis no longer can properly metabolize intravenously administered fat (3). However, no data are available to indicate whether or not these patients likewise might suffer from any impaired ability to absorb fat from their gastro-intestinal tracts. Should such an impairment exist, then the benefit which patients with hepatic disease would derive from ingested fat must be very limited. To provide, in part, some answer to these questions of fat absorption, 2 patients with hepatic cirrhosis were studied. One of these was a chronic alcoholic and the diets of both had been grossly deficient in animal protein but not in fat.

While on the basal diet for from 3 to 6 days, the stearin excreted by these 2 individuals ranged from 4.9 to 9.5 grams per day. These outputs accounted for from 2 to 8.1 per cent of the fat ingested, or indicated an absorption of from 92 to 98 per cent of the fat from their gastro-intestinal tracts.

The addition of the fat supplement to their diets increased the fecal fat excretions to from 2.4 to 8.8 grams per day. These values represented an absorption of from 93 to 97 per cent of the supplements. Thus no significant defect appeared

to exist in the absorption of fat from the gastro-intestinal tract of the patients with hepatic cirrhosis included in this study.

#### DISCUSSION

The results of the present investigation demonstrate the efficiency of fat absorption from the gastro-intestinal tract of normal individuals, of patients with hepatic cirrhosis, and of a patient with gastric cancer. The percentage values obtained for the absorption of ingested fat probably are slightly higher than the true value, for no correction has been made for the small amounts of fecal lipid which is known to be of endogenous origin. Ordinarily, this endogenous fat varies from 0.2 to 3.0 grams per day (8), which would indicate that only from 1 to 5 grams of the fecal lipid excreted by this group of subjects who ingested 1.5 grams of fat per kilogram of body weight, was of dietary origin.

It is worthy of note that the addition of large amounts of fat to the diet of normal subjects, of the individual with gastric cancer, and of the patients with hepatic cirrhosis, failed to produce a striking rise in the output of fecal fat. Previous studies have demonstrated a considerable degree of physiologic hepatic dysfunction both in patients with gastric cancer (9) and in those with portal cirrhosis (10). It is, therefore, interesting to observe that hepatic dysfunction apparently plays no constant, significant role in the absorption of fat from the gastro-intestinal tract.

Finally, increased intestinal motility must be considered a causative factor for the steatorrhea. To exclude this possibility, studies were carried out by means of barium and fluoroscopic roentgenography and the marking of diet periods with carmine and charcoal. These studies indicated no significant departure from the normal. Thus, it would appear that increased intestinal motility was not a causative factor for the steatorrhea.

However, the possibility must be considered that the proper absorption of lipids is influenced by the presence of a normal, functioning gastric mucosa. Both the patient with severe atrophic gastritis, and the individual who lacked a stomach entirely, suffered from a decreased efficiency of fat absorption. The nature of this defect, and the

measures necessary for its effective treatment, will be presented in a subsequent report.

### CONCLUSIONS

1. The gasometric method of Van Slyke *et al.* was adapted to measure the absorption of fat from the gastro-intestinal tract of a group of subjects. This included 2 normal individuals, 1 patient bearing gastric carcinoma, 1 patient who had undergone total gastrectomy, 1 patient with generalized atrophic gastritis, and 2 patients with hepatic cirrhosis.

2. An abnormal absorption of fat was demonstrated only in the gastrectomized patient and in the patient with atrophic gastritis.

3. The question is raised of a relationship between the absence of an intact gastric mucosa and the normal absorption of fat from the gastro-intestinal tract.

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# SULFONAMIDE-FAST PNEUMOCOCCI. A CLINICAL REPORT OF TWO CASES OF PNEUMONIA TOGETHER WITH EXPERIMENTAL STUDIES ON THE EFFECTIVENESS OF PENICILLIN AND TYROTHRICIN AGAINST SULFONAMIDE-RESISTANT STRAINS

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Demonstration of the fact that strains of pneumococci may become resistant to the antibacterial action of the sulfonamide drugs has emphasized one of the potential difficulties that may arise in the chemotherapy of pneumonia. Not only has the acquisition of drug-fastness been induced by laboratory procedures (1 to 3) but individual strains of pneumococci obtained from patients have exhibited the capacity to change within the body from susceptible to resistant states during the course of treatment (4 to 7).

The findings recorded in this report were derived from observations on two patients who had pneumococcus pneumonia with bacteremia and who failed to respond to sulfadiazine but improved rapidly following serum therapy. Clinical and laboratory data are presented which indicate that the infections were caused by drug-resistant pneumococci. In addition, the patients' strains have been used for experimental studies with particular reference to problems relating to treatment. For this purpose, infected mice have been treated with penicillin and with tyrothricin, as well as with sulfadiazine, in order to compare the curative action of the three agents.

## CLINICAL AND LABORATORY DATA OF THE PATIENTS

The pertinent findings in the course of the diseases in the two patients are given in Figure 1 for patient M. R., who had Type I pneumococcus pneumonia, and in Figure 2 for patient W. L., who had Type VIII pneumococcus pneumonia (Figure 1).

Patient M. R. was a white female, aged sixty-two years. She was admitted on the fourth day of the disease and was found to be seriously ill with pneumonic consolidation of the right lower and middle lobes. The leukocyte count was 9,900. There was no sputum. Blood culture was positive for pneumococcus Type I. Sulfadiazine was administered on the first hospital day, both intravenously and by mouth. From Figure 1, it may be noted

that her temperature dropped to 100° F., on the third hospital day. However, clinical evidence of improvement did not parallel the temporary fall in temperature. On the fourth hospital day, the fever had risen again above 102° F.

Pneumococcus Type I was obtained from a throat culture taken on the seventh hospital day. A second blood culture, taken on the eighth hospital day, was also positive for pneumococcus Type I.

The patient remained acutely ill and bacteremia persisted in spite of the maintenance of blood levels of sulfadiazine ranging from 9.1 to 13.4 mgm. per cent. Consequently Type I antipneumococcus rabbit serum was administered as indicated in Figure 1. The drop in the temperature was attended with marked clinical improvement. Subsequent blood cultures were sterile. The patient recovered uneventfully.

From the course of the disease in this patient, both with respect to the persistence of signs and symptoms of acute illness as well as the bacteremia, it is evident that sulfadiazine was not effective even though the disease was limited to a primary uncomplicated pneumonia. Although the first two blood cultures were taken a week apart, the absence of any clinical improvement during the first eight days is strongly suggestive that the bacteremia may have been continuously present. Furthermore, the simultaneous presence in the blood stream of both pneumococci and appreciable levels of sulfadiazine indicate the absence of antibacterial action of the drug on the infecting organisms. When these findings are correlated with the failure of sulfadiazine, as shown later on, to cure mice infected with the strain of pneumococci derived from this patient, the conclusion seems clear that the drug-fast characteristics of the infecting organisms accounted, in all probability, for the patient's lack of response to chemotherapy.

The strain of pneumococcus Type I from patient M. R. which was subsequently used for laboratory tests was isolated from a throat culture taken on the seventh hospital day. Conclusive evidence, therefore, of the nature of the strain before the beginning of treatment was not determined. However, the persistence of active illness, in spite of adequate administration of sulfadiazine, suggests that the infecting organism was either resistant from the beginning or had quickly acquired the property.

Patient W. L. (Figure 2) was a colored male, aged 50



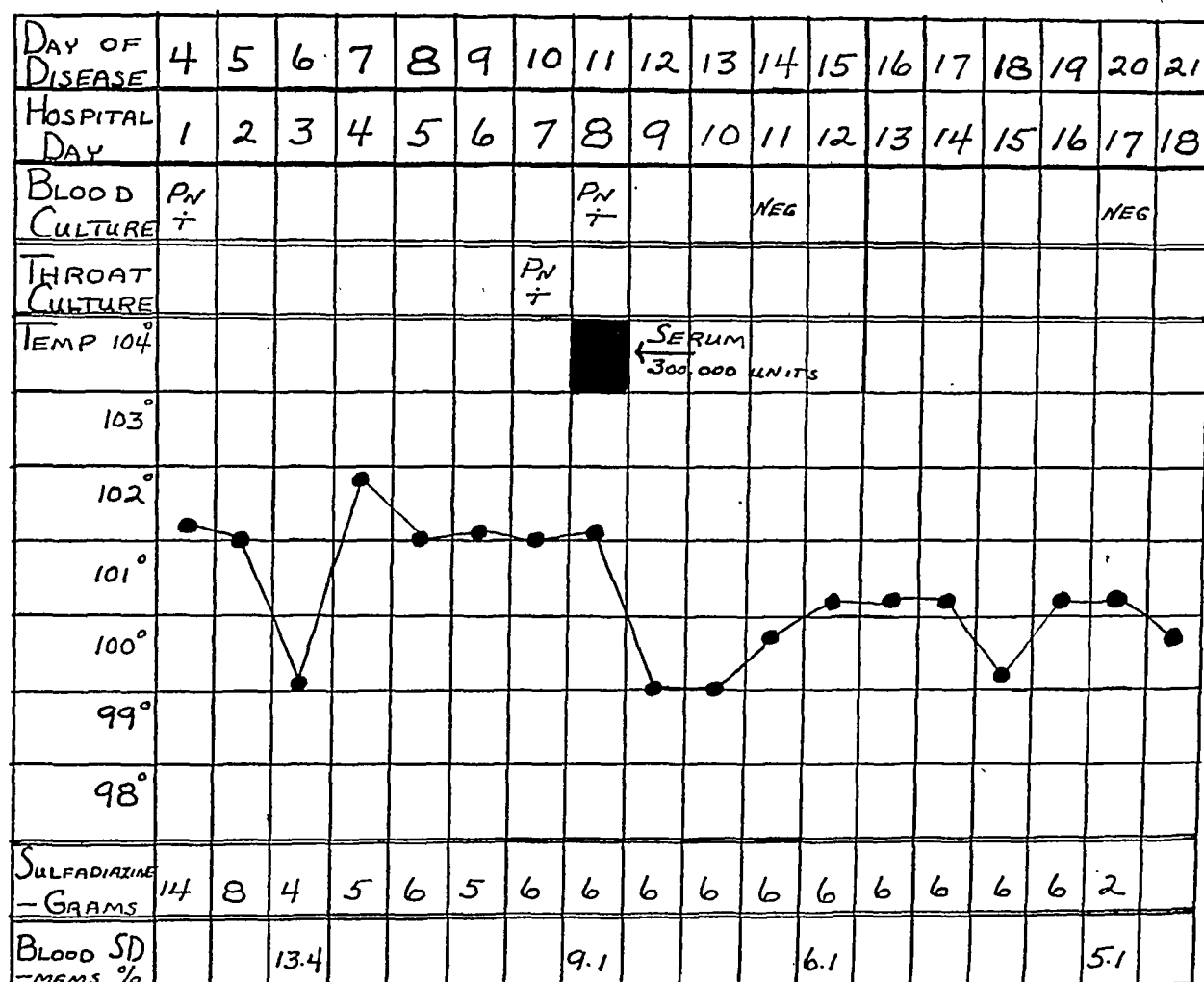


FIG. 1. PATIENT M. R.; TYPE I PNEUMOCOCCUS PNEUMONIA

years. He was admitted the day after onset of pneumonia which began with chill and sharp pain in left chest.

On admission, there were signs of early pneumonia in the left upper lobe. The leukocyte count was 9,000. *Pneumococcus* Type VIII was present in sputum and also in the first blood culture. The Wassermann reaction was positive.

Chemotherapy was begun on admission with sulfathiazole, but later changed to sulfadiazine. There was no improvement in the first three days and, as indicated in Figure 2, the temperature increased on the fourth hospital day. Daily blood cultures were positive for the first five days. Signs of a small pleural effusion were present on the fifth day. Five to 10 cc. of thin cloudy fluid were obtained by thoracentesis, a culture of which yielded *pneumococcus* Type VIII. The sulfonamide level in the pleural fluid was 4.3 mgm. per cent. This strain was used in laboratory studies, the results of which are presented in Table I and discussed later.

Antipneumococcus rabbit serum, administered on the fifth and sixth hospital days, brought about marked clinical improvement. Subsequent blood cultures were sterile. The small encysted empyema persisted. Numerous subsequent attempts were made to obtain fluid but in only one instance, on the eighteenth day, were 2 to 3 cc.

of a bloody exudate obtained. *Pneumococcus* Type VIII was present in the specimen. The area subsequently resolved and the patient was discharged from the hospital on the sixtieth day.

As noted in Figure 2, pneumococci and levels of sulfonamide of from 5.8 to 7.6 mgm. per cent were present concurrently in the blood stream. Although the organisms of the blood cultures were not tested, the first culture of the pleural fluid yielded *pneumococcus* Type VIII, which, when used for infection in mice, proved refractory to treatment with sulfadiazine. The course in this case, on the basis of a persistent bacteremia as demonstrated by daily blood cultures, suggests, perhaps more definitely than the first case, the probability that the infecting strain was drug-fast from the beginning of the illness.

#### DISCUSSION

A review of the literature indicates that up to the present time the incidence of cases of pneumonia caused by drug-fast strains is a small fraction of the total. Ross (4) reported a strain from a fatal case of meningitis which, by cultural tests, was susceptible to sulfonamide bacteriostasis early

in the disease but a second culture obtained at autopsy was drug-resistant. Hamburger and associates (7) described a strain of pneumococcus from a case of bacterial endocarditis which, following prolonged chemotherapy, acquired drug-resistant characteristics, as demonstrated *in vitro*. Other observers, in studying strains derived from cases of pneumonia, have noted variations in the capacity of the organism to multiply in culture media containing stated quantities of sulfonamide drugs. In early cases of uncomplicated pneumonia, Lowell, Strauss, and Finland (6) did not encounter resistant strains among those isolated before treatment was begun. In three instances, they observed strains that were relatively resistant after a few days of chemotherapy. In some patients who failed to respond to drug treatment, however, no change in the susceptibility of the strains, which might account for the unsatisfactory results, was noted. Cotler, Kirchner, and Romano (8) cultivated specimens of material from

cases of pneumonia on blood agar containing varying amounts of sulfapyridine. They cited five cases in which the lack of therapeutic effect of the drug was particularly striking. In three of the instances, the strains were drug-resistant in that colonies developed on blood agar containing sulfapyridine. By testing pneumonic sputum injected into treated mice, Moore, Thomas, and Hoyt (9) found one strain of pneumococcus, out of thirty-three derived from different patients with pneumonia, to be sulfonamide resistant.

In a recent report, Tillett (10) noted that during a two-year period of observation on chemotherapy in pneumonia, pneumococcus bacteremia was found to persist for as long as seven days only in cases complicated by endocarditis. The two cases of the present report are obvious exceptions to the earlier experience.

A noteworthy feature of the clinical course of the two patients described in the present report was the rapid and satisfactory response to serum

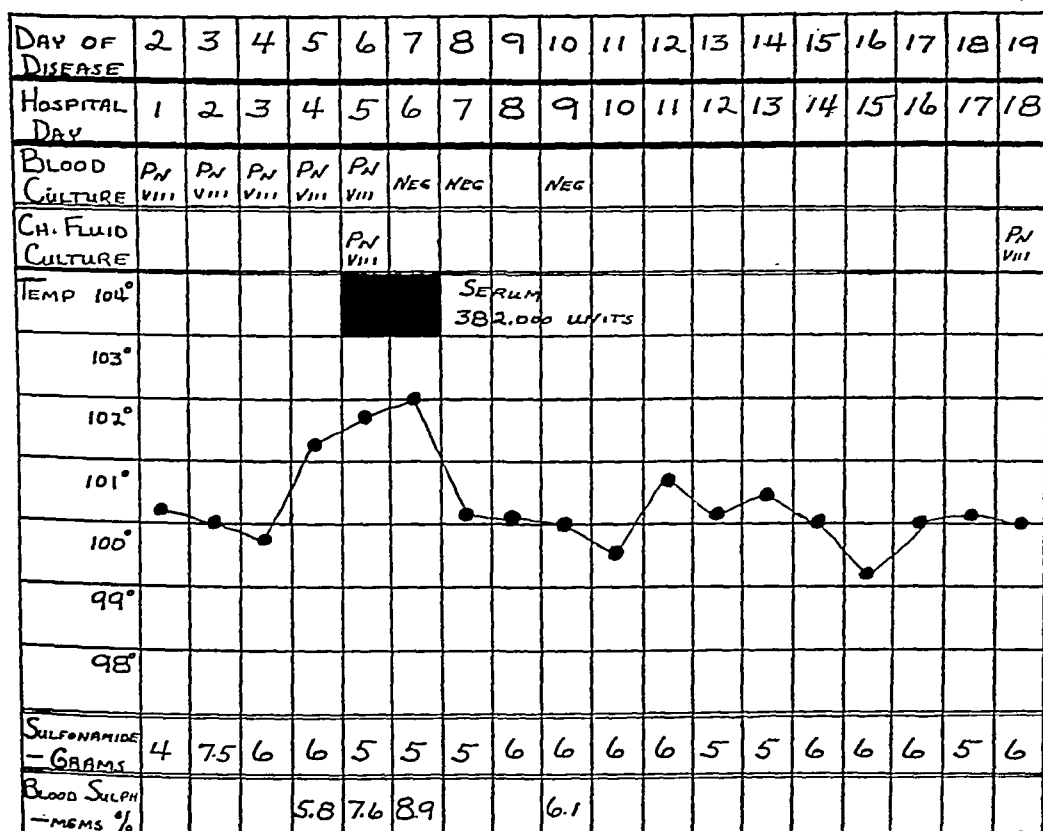


FIG. 2. PATIENT W. L.; TYPE VIII PNEUMOCOCCUS PNEUMONIA

therapy. MacLeod and Daddi (2) found infections in mice, produced by the resistant strain of pneumococcus Type I which they employed, to be just as amenable to cure with antipneumococcus serum as were infections with susceptible strains of Type I. Lowell, Strauss, and Finland (6) observed rapid improvement in two patients who were treated with serum following a relapse of pneumonia which developed during sulfapyridine therapy. The strains from their two cases were found by cultural methods to be drug-resistant.

*Experimental studies with patients' strains of pneumococci Types I and VIII in relation to therapeutic use of sulfadiazine, penicillin, and tyrothricin*

The sources of four strains of pneumococci used in the tests were as follows: Type I resistant strain came from a throat culture taken on patient M. R. on the seventh hospital day. Type VIII resistant strain came from the pleural fluid of patient W. L. obtained on the fifth hospital day. Type I and Type VIII susceptible strains were stock laboratory cultures.

Infections in mice were produced with each of the four strains by intraperitoneal injection of graded amounts of the respective cultures.

The preparation of the material and the methods of treatment with sulfadiazine, penicillin, and tyrothricin were as follows:

**Sulfadiazine.** The drug in powder form was suspended in gum tragacanth. Treatment was given *per os* by means of a small silver catheter used as a stomach tube. A dose of 20 mgm. per treatment was most regularly employed, twice on the first day and once daily thereafter for three to four days. The amount of drug for a single dose was contained in 0.5 cc. of the mixture of sulfadiazine and tragacanth. The infecting dose of culture was injected five to ten minutes after the first oral administration.

**Penicillin.** The powdered material was received in sealed ampoules.<sup>1</sup> Each ampoule contained 5,000 so-called Florey units (11). The contents of a single ampoule were dissolved in 10 cc. of distilled water which yielded a clear yellowish solution containing 500 units per cc. The data presented in Table I consist of the combined results which were obtained when either 0.5 cc.

of the original solution (250 units) or 0.5 cc. of a 1-10 dilution (25 units) were employed. For the data given in Table I, mice received penicillin three times daily, by the intraperitoneal route, for four days. The first injection was given ten to fifteen minutes after the infection.

**Tyrothricin.** The material was made available by Dr. Colin MacLeod who obtained it from Merck & Company. The term tyrothricin was applied by Dubos and Hotchkiss (12) to material which contains both gramicidin and tyrocidin. Although more toxic than gramicidin, the curative action of tyrothricin for experimental pneumococcal infections has been established (12).

The powder was dissolved in absolute alcohol, in sufficient quantity to make a 4 per cent solution. Additional dilutions were made in distilled H<sub>2</sub>O so that 1 cc. contained either 0.04 mgm. or 0.08 mgm. Each mouse received, as the total treatment, one single dose of 0.5 cc. (0.02 mgm. or 0.04 mgm.) intraperitoneally, within a few minutes after the infection.

The combined results of different experiments concerning the curative action of the three reagents, sulfadiazine, penicillin, and tyrothricin, for the four strains of pneumococci are contained in Table I.

From Table I, it may be noted that no therapeutic effect was obtained with sulfadiazine against the two patients' strains, even when the amount of infecting inoculum was as small as  $10^{-6}$  dilution of culture. Against the two stock strains, however, effective cure was uniformly obtained against  $10^{-2}$  cc. of culture.

The negative therapeutic results with the resistant strains were obtained in the first tests performed within a few days after their isolation and also in similar tests made three months later. The persistence of the drug-fast qualities by resistant strains, noted in the cultures used in the present experiments, has also been reported by other investigators (2).

In contrast to the selective results obtained by treatment with sulfadiazine, when penicillin was employed the curative action was equally great against all four strains. The data in Table I demonstrate that the mice uniformly survived, whether they received maximum doses ( $10^{-2}$  cc) of sulfonamide-resistant strains or comparable amounts of susceptible strains.

<sup>1</sup> Penicillin was obtained from E. R. Squibb and Son.

TABLE I

*Therapeutic action of sulfadiazine, penicillin, and tyrothricin in mice infected with patients' strains and stock strains of pneumococci Types I and VIII*

Cultures		Sulfadiazine			Penicillin			Tyrothricin		
Strain	Amount of inoculum	Number mice	Number survivals	Per cent survivals	Number mice	Number survivals	Per cent survivals	Number mice	Number survivals	Per cent survivals
Type I M. R. (patient)	$10^{-2}$	4	0	0	6	6	100	8	6	75
	$10^{-3}$	6	0	0	8	8	100	8	7	87
	$10^{-4}$	3	0	0						
	$10^{-5}$	6	0	0						
	$10^{-6}$	8	0	0						
Type I (Stock)	$10^{-2}$	6	6	100	5	5	100	8	7	87
	$10^{-3}$	5	5	100	6	6	100	8	8	100
	$10^{-4}$	3	3	100						
	$10^{-5}$	3	3	100						
	$10^{-6}$	3	3	100						
Type VIII W. L. (patient)	$10^{-2}$	4	0	0	6	6	100	8	7	87
	$10^{-3}$	10	0	0	10	10	100	8	8	100
	$10^{-4}$	4	0	0						
	$10^{-5}$	6	0	0						
	$10^{-6}$	8	0	0						
Type VIII (Stock)	$10^{-2}$	5	5	100	5	5	100	8	2	25
	$10^{-3}$	10	10	100	5	5	100	8	6	75
	$10^{-4}$	5	5	100						
	$10^{-5}$	5	5	100						
	$10^{-6}$	5	5	100						

In control mice, used in each separate experiment, an inoculum of  $10^{-6}$  of culture of each strain proved uniformly fatal.

Fleming (13) who first described the action of penicillin, found that the material inhibited the growth of gram positive cocci. Subsequently Chain, *et al.* (11), Florey, *et al.* (14), and Hobby, Meyer, and Chaffee (15) have demonstrated *in vivo* activity against pneumococci. Powell and Jamieson (16) have found penicillin to be effective against sulfonamide-fast strains of pneumococci. McKee and Rake (17), in a brief report, state that mice infected with sulfonamide-resistant strains of pneumococci have been protected with penicillin. Florey and Jennings (18) remarked upon the fact that sulfonamide-resistant strains of streptococci were sensitive to penicillin.

When tyrothricin in single doses per mouse of 0.02 mgm. or 0.04 mgm. was used for treatment, the extent of protection afforded the mice was found in different experiments to be variable. In comparing the results (Table I) obtained with each of the four strains, it may be noted, however, that there was no evidence of special refractoriness exhibited by the mice infected with the sulfonamide-resistant strains. On the contrary, among the infections caused by the four strains, tyrothricin was found to be least effective against

one of the stock strains (Type VIII) which is sulfonamide susceptible. Consequently, the findings have failed to indicate any relationship between sulfonamide resistance and factors which influence the effect of tyrothricin on pneumococci.

#### *Tests for the production of sulfonamide inhibiting substances by strains of pneumococci*

Repeated studies have demonstrated that the resistance of bacteria to the action of the sulfonamides is not infrequently associated with the production of inhibiting substances by the refractory strains. MacLeod (19) reported results obtained with a strain of pneumococcus which was made sulfonamide-fast *in vitro*. The strain was found to produce significant amounts of inhibitor. The same author has also referred to other resistant strains of pneumococci which, however, did not produce demonstrable amounts of anti-sulfonamide substances (20).

Both the resistant and susceptible strains used in the present study have been tested for the capacity to produce inhibitor. As a positive control of the technique employed, the resistant Type I strain of MacLeod was also used in comparative tests.

The tests were carried out according to the procedure reported by MacLeod (19), using a strain of *B. coli* cultivated in the special media described by Sahyun, Beard, Schultz, Snow, and Cross (21). Into the tubes containing 5.0 cc. of basic media, serial dilutions of sulfadiazine were added. The strain of *B. coli* grew in the presence of 1 part in 2,000,000 (equivalent to 0.0025 mgm.) of sulfadiazine but was inhibited by 1 part in 1,000,000 (0.005 mgm.) of the drug.

The five strains of pneumococci used in the tests were first cultivated in the synthetic media described by Bernheimer, Pappenheimer and associates (22). In preliminary trials, sterile uninoculated preparations of the media were found to be devoid of inhibitor commonly encountered in broth. Five tenths cc. of the supernatant fluid of cultures of pneumococci was added to the basic materials, and the tubes were inoculated with  $10^{-4}$  dilution of *B. coli*. Since the pneumococci were unable to grow in the media of Sahyun, Beard, *et al.*, either with or without *B. coli*, it was unnecessary to heat the cultures of pneumococci before adding the supernatant fluid to the other elements of the test. The results are given in Table II.

From the data of Table II, it may be seen that a moderate but equal amount of inhibiting substance was developed by both the sulfonamide-fast patient's strain of Type I and the sulfonamide-susceptible stock Type I. Consequently, on the basis of the test, the two strains could not be differentiated. However, with the drug-fast Type I strain of MacLeod, the production of inhibitor was greatly increased over that of the other strains.

With the Type VIII cultures, the yield of inhibitor by the sulfonamide-susceptible stock strain was comparable to that of the Type I stock strain. Cultures of the sulfonamide-resistant strain from patient W. L. were the weakest of any of the strains in the production of inhibiting substances.

On the basis, therefore, of the findings summarized in Table II, it has not been possible to ascribe the resistance of the patients' strains to the production of sulfonamide inhibiting substances. Furthermore, the results obtained with patient's Type I strain and with MacLeod's Type I strain appear significant in indicating that among strains of pneumococci, even of the same type, different mechanisms may exist for nullifying the action of the sulfonamide drugs.

TABLE II  
*Tests for production of inhibitor by five strains of pneumococci*

Supernatant fluid	Dilutions of sulfadiazine in synthetic media*					
Strain	1 to 20,000	1 to 50,000	1 to 100,000	1 to 500,000	1 to 1,000,000	1 to 2,000,000
Type I M. R. (patient)	—	—	—	+++	++++	++++
Type I (Stock)	—	—	—	+++	++++	++++
Type I (MacLeod)	+++	++++	++++	++++	++++	++++
Type VIII W. L. (patient)	—	—	—	—	—	++++
Type VIII (Stock)	—	—	—	+++	++++	++++
Sterile Pneumococcus Media†	—	—	—	—	—	++++
Control	—	—	—	—	—	++++

+++ , ++++ indicate visible density of growth of *B. coli*, — indicates no visible growth. Final readings of growth were made after 48 hours incubation. \* Sahyun, Beard, *et al.* (21). † Bernheimer, Gillman, *et al.* (22).

## SUMMARY

1. From two patients having pneumonia and bacteremia, strains of pneumococci (Types I and VIII) were isolated which caused infections in mice, that were totally refractory to treatment with sulfadiazine.

The clinical course of the patients and the clinical laboratory data (blood cultures and levels of sulfonamides in blood) also indicated the drug-resistant characteristics of the infection.

Both patients responded rapidly and successfully to specific serum therapy.

2. In experimental observations, penicillin was found to be highly effective against infections in mice caused by either sulfonamide-resistant or susceptible strains.

When tyrothricin in single doses was used for treatment of mice, the protection was not uniformly complete against any one of the strains. However, no evidence was obtained to indicate that sulfonamide resistance influenced the effect of tyrothricin.

3. It was not possible to demonstrate that the drug-resistance of the patients' strains was due to the production of inhibiting substances. By contrast, cultures of an additional strain of Type I pneumococcus (MacLeod) were found to yield considerable amounts of inhibitor.

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# NITROGEN STORAGE FOLLOWING INTRAVENOUS AND ORAL ADMINISTRATION OF CASEIN HYDROLYSATE TO INFANTS WITH ACUTE GASTRO-INTESTINAL DISTURBANCE<sup>1</sup>

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The acute gastro-intestinal disturbances of infants have always been a source of anxiety to the pediatrician. Nutritional therapy has made severe demands upon his skill. The two horns of the dilemma are, first, that any kind of food by mouth may lead to increase of untoward symptoms already present, *i.e.* diarrhea and vomiting; and second, that deprivation of food for a relatively short time may lead to collapse. The accompanying dehydration and acidosis are complications which often cause a fatal outcome. We have learned, over the course of the last few decades, how to replenish the interstitial fluids, to overcome the acidosis, and to provide for a substantial portion of the caloric requirement by parenteral therapy. This, coupled with the use of sulfonamides when indicated, constitutes such great improvement in therapy that modern clinicians with good hospital facilities no longer have the same dread of these diseases as those of a generation ago.

But these procedures still do not provide nitrogen, which is essential for normal nutrition. Protein or its equivalent could, until fairly recently, be supplied only by intravenous plasma infusions. Another source of nitrogen is now available for intravenous use,—protein hydrolysate. Such a preparation has already been shown to be an adequate source of nitrogen for the nutrition of infants who are not ill, whether given orally or intravenously (1).

The question arose as to how large a nitrogen loss infants with acute gastro-intestinal disorders suffer, and whether amino acid therapy would change a negative to a positive balance. The purpose of this study was to make a quantitative determination of the effect upon nitrogen balances

of enteral and parenteral administration of casein hydrolysate to infants suffering from acute gastro-intestinal symptoms. In order to avoid undue complexity, analysis of the clinical aspect is included only in so far as it is pertinent.

## EXPERIMENTAL

The 20 male infants included in this study were patients sent to the hospital because they were too ill to be treated as out-patients. The main features of the illnesses were vomiting, diarrhea, and fever, with or without infection, which led to dehydration and sometimes acidosis of such a grade as to require parenteral fluid therapy and withholding of food by mouth. This group did not include any patients with specific intestinal infections such as dysentery or typhoid fever. The infants were from 2 weeks to 8 months of age, and from 2.6 to 9 kgm. in weight.

Saline solution was given intravenously to replenish depleted body fluids. Glucose-lactate solution was given when necessary to alleviate acidosis. Nitrogen was provided as casein hydrolysate, given intravenously or orally. Specific drugs were given as indicated.

Some of the infants were selected for this study immediately following admission to the hospital; others were given fluids parenterally, but no nitrogen, for 1 or 2 days before administration of casein hydrolysate was begun. Blood transfusions were given in several cases before and during the time of casein hydrolysate administration.

The preparation used for intravenous administration was an enzymic hydrolysate of casein plus glucose in solution.<sup>2</sup> This was sometimes modified in our laboratory, but contained, as given, 2.5 to 4 per cent casein hydrolysate, 5 to 10 per cent glucose, and varying amounts of NaCl. We also used, for oral administration, a prepared food containing casein hydrolysate,<sup>3</sup> which we had

<sup>2</sup> This material, the trade name of which is "Amigen," was prepared and supplied through the courtesy of Mead Johnson and Co. Amigen contains no unhydrolyzed protein. The nitrogen is present in various forms; about 60 per cent is amino nitrogen.

<sup>3</sup> This was a mixture of 20 per cent casein hydrolysate (Amigen), 42.3 per cent dextrin-maltose, 10 per cent starch, 18 per cent olive oil, 3 per cent brewers' yeast,

<sup>1</sup> This investigation was aided by a grant from Mead Johnson and Co.



used in previous studies (1), and which had been found to be adequate to meet nutritional needs of infants for protracted periods (2).

Each of the infants was placed upon a metabolism bed. When there was more than one period of observation, they were consecutive. Most of the periods were of 3 days' duration. Some as short as 24 hours were periods during which intravenous therapy only was given, and were short because of change either in clinical condition or in type of therapy. The urine and feces were collected and analyzed separately for nitrogen by the micro Kjeldahl method. The nitrogen intake also was determined by analysis.

The plan was to omit food by mouth and to give casein hydrolysate-glucose-saline solution intravenously for 24 to 48 hours, or until the vomiting ceased and the diarrhea subsided. The second phase of the treatment was to give the same or similar solution orally instead of, or in addition to, intravenously. If this form of treatment was well tolerated, the third phase was begun, namely, the casein hydrolysate food (containing starch and fat) or milk formula was given instead of, or in addition to, the casein hydrolysate solution. In a few cases, we have continuous observations on these three phases of treatment. Inasmuch as no two clinical cases are exactly alike, such a division is arbitrary and represents a clinical judgment in each case rather than a well defined division into stages of progress toward recovery. The phases of treatment correspond roughly with the stages of improvement in clinical condition. When recovery was sufficiently advanced, the infant was returned to milk formula feeding. In conformity with the accepted procedure, smaller amounts of material were given when the symptoms were most acute, and quantities were increased as the patient's condition improved. The caloric intake amounted to 40 to 90 Calories per kgm. of body weight per day. These values are below the optimal for normal nutrition, but higher than usually given in these cases. In general, the lower caloric intakes were associated with the lower nitrogen intakes and the two increased together.

The solution was given intravenously, by constant drip through a No. 22 or No. 24 needle, into a scalp, arm, or leg vein, and allowed to flow by gravity at the rate of about 20 drops per minute, or 50 cc. per hour. Due largely to procedures which we no longer regard as correct, local edema and erythema developed in about one fourth of the patients. In every case, the edema subsided within a few hours after the needle was removed. It tended to be more severe when the injection was continued more than 10 or 12 hours, and when the lower extremities were used. The solution may have leaked through the puncture holes in the veins. Further, intact

and 6.7 per cent salts. This product was prepared by and supplied through the courtesy of Mead Johnson and Co. A Mead Johnson product is now available under the trade name of "Nutramigen" which is identical with the preparation we used except that it contains corn oil instead of olive oil.

vessels which would prevent the passage of plasma protein would permit permeation of the small molecules of amino acids. If the amino acids spread into the tissues, there is every reason to believe that they were subsequently reabsorbed and utilized. In addition, as mentioned above, our solutions contained NaCl above that present in the casein hydrolysate. This led to increased water retention as is evidenced by the weight curves of the infants. The amount of NaCl present in the original product is sufficient to assure proper hydration of the tissues, and the excess merely tended to increase the local edema. Since we have abandoned the use of extra NaCl and have decreased the time of injection to less than 8 hours, we are no longer troubled with the edema which we encountered in these studies.

The temperature elevations accompanying intravenous administration of casein hydrolysate, which were reported previously, were not prominent in a single case, and were either absent or negligible. No injection had to be interrupted for this cause. This was probably due to an improvement in the preparation of the material. Extra precautions were taken so that the water used was free of pyrogenic material.

In this series, all the infants, with 2 exceptions, made good recoveries. It is our impression that the improvement was as good or better than could be obtained without amino acid therapy. Two died, W. McG. of bacterial pericarditis and S. K. of septicemia, diseases which they had when they were admitted to the hospital. Diarrhea was in both cases a presenting symptom. Of the others, except for S. P., who had a "break" in the last period, with vomiting and diarrhea, all the patients made more or less uneventful progress.

Observations were made upon 4 infants with approximately the same type and degree of illness, who did not receive amino acid therapy. Two (T. S. and J. T.) were given the usual glucose and saline parenterally, and 2 (R. F. and J. W.) received milk formulas by mouth. These served as controls.

## RESULTS AND DISCUSSION

The data for nitrogen intake, output, and balance are summarized in Table I. The details of the composition and concentration of injected and ingested material, length of periods, rate of intravenous administration, and volume of intake and output are not included, as they are similar to cases already reported (1, 3) and do not give any information necessary to this exposition. In order that the data on infants of different sizes and on periods of different lengths may more readily be compared, all results have been expressed as grams of nitrogen per day (24 hours) or as grams per kgm. of body weight per day. For approximation data, calculation of metabolism on the basis of body weight is simpler than on the

TABLE I  
*Nitrogen metabolism of infants with acute gastro-intestinal disturbance*

Subject			Intake*				Output		Balance		
Name and age	Phase†	Weight‡	Material	Method	N per day	N per kgm. per day	Urine	Feces	N per day	N, per cent of intake	N per kgm. per day
R. C. 8 months	I	kgm. 8.30	cas. hyd.	i.v.	grams 5.50	grams 0.67	grams 2.85	grams 0.01	grams 2.64	48	grams 0.32
	II	8.40	cas. hyd.	{ i.v. oral	0.60 2.40	} 0.36	1.37	0.15	1.48	50	0.18
	II	8.60	cas. hyd. blood	{ i.v. oral i.v.	3.86 0.11 3.07	} 1.28	3.04	0	4.00	57	0.47
	III	9.00	cas. hyd. c.h.f. blood	i.v. oral i.v.	2.32 1.00 1.60	} 0.54	1.33	0.23	3.36	68	0.37
	III	9.60	c.h.f.	oral	2.85	0.30	1.45	0.48	0.93	33	0.10
S. C. 3 months	I	3.14	cas. hyd.	i.v.	1.53	0.49	0.74	0.05	0.74	48	0.24
	II	3.17	cas. hyd.	{ i.v. oral	0.10 2.52	} 0.80	0.81	0.34	1.47	56	0.47
	III	3.20	cas. hyd. c.h.f.	oral oral	1.76	0.55	0.94	0.10	0.72	41	0.22
D. D. 2 weeks	I	2.70	cas. hyd.	i.v.	1.48	0.55	0.56	0.27	0.65	43	0.24
	III	2.85	cas. hyd. c.h.f. blood	oral oral i.v.	0.27 0.97	} 0.44	0.55	0.26	0.43	34	0.15
	III	3.00	c.h.f.	oral	1.33	0.44	0.37	0.31	0.65	49	0.21
R. McK. 1 month	II	2.85	cas. hyd. blood	oral i.v.	1.05 0.81	} 0.66	0.31	0.32	1.23	66	0.43
	I	2.79	cas. hyd. blood	i.v. i.v.	1.88 1.54	} 1.22	0.68	0.28	2.46	72	0.88
	I	2.95	cas. hyd.	i.v.	3.16	1.07	1.07	0	2.09	66	0.71
	II	3.00	cas. hyd.	oral	1.59	0.53	0.45	0.15	1.14	72	0.38
	II	3.00	cas. hyd. blood	oral i.v.	1.40 1.75	} 1.05	0.60	0.15	2.40	77	0.80
	III	3.16	c.h.f.	oral	1.20	0.38	0.62	0.21	0.37	31	0.12
S. P. 1 month	I	3.24	cas. hyd.	i.v.	2.60	0.80	0.88	0.16	1.56	60	0.48
	II	3.24	cas. hyd.	{ i.v. oral	0.18 2.25	} 0.75	0.85	0.51	1.07	44	0.33
	III	3.30	c.h.f. blood	oral i.v.	0.60 2.17	} 0.84	0.55	0.60	1.62	59	0.49
	III	3.30	c.h.f.	oral	0.90§	0.27	0.95	0.29	-0.34		-0.10
E. P. 6 months	I	6.00	cas. hyd.	i.v.	3.10	0.51	3.31	0.40	-0.61		-0.10
	II	6.00	cas. hyd.	{ i.v. oral	0.28 5.46	} 0.96	2.11	0.27	3.36	58	0.56
	II	6.40	cas. hyd.	oral	5.58	0.87	3.48	0.30	1.80	32	0.28
	III	6.70	cas. hyd. c.h.f.	oral oral	3.78	0.56	2.94	0.32	0.52	14	0.08
	III	6.50	c.h.f.	oral	1.75	0.27	1.70	0.27	-0.22		-0.03

TABLE I—Continued

Subject			Intake*				Output		Balance		
Name and age	Phase†	Weight‡	Material	Method	N per day	N per kgm. per day	Urine	Feces	N per day	N, per cent of intake	N per kgm. per day
		kgm.			grams	grams	grams	grams	grams		grams
W. McG. ¶ 5 weeks	III	3.16	fat-free milk + glucose	oral	1.27	0.42	0.49	0.24	0.54	42	0.17
	I	2.83	cas. hyd.	i.v.	2.37	0.84	1.27	0.06	1.04	43	0.37
J. J.	I	6.90	cas. hyd.	i.v.	2.80	0.41	2.65	0.45	-0.30		-0.04
A. B. 3 weeks	I	3.26	cas. hyd.	i.v.	2.08	0.64	1.35	0.26	0.47	23	0.14
	I	3.10	cas. hyd.	i.v.	1.67	0.54	1.04	0.11	0.52	31	0.17
S. K. ¶ 4 weeks	I	2.64	cas. hyd.	i.v.	1.57	0.60	1.27	0.04	0.26	17	0.10
W. T. 5 months	I	5.33	cas. hyd.	i.v.	2.50	0.47	2.05	0.33	0.12	4	0.02
J. diB. 7 weeks	II	3.69	cas. hyd.	{ i.v. oral	0.89 0.19	} 0.29	0.71	0.05	0.32	30	0.09
R. H. 3 months	II	4.90	cas. hyd.	{ i.v. oral	2.34 0.39	} 0.56	2.05	0.06	0.62	25	0.13
J. F. 3 weeks	II	3.00	cas. hyd.	{ i.v. oral	1.75 1.35	} 1.03	0.73	0.12	2.25	72	0.75
	III	3.23	c.h.f.	oral	1.62	0.50	0.37	0.05	1.20	73	0.37
A. W. 18 months	III	9.90	cas. hyd. c.h.f.	i.v. oral	1.41 3.33	} 0.48	1.70	0.74	2.30	49	0.23
	III	10.00	cas. hyd. c.h.f.	i.v. oral	1.00 3.05	} 0.40	2.32	1.36	0.37	9	0.04
J. D. 1 month	I	3.22	cas. hyd.	i.v.	2.08	0.64	0.92	0.06	1.10	53	0.34

## Controls

T. S. 5 weeks	I	2.87	glucose + saline lactate	i.v.	0	0	0.61	0.12	-0.73		-0.25
J. T. 6 months	I	6.85	glucose + saline	i.v.	0	0	1.15	0.25	-1.40		-0.21
		6.85	glucose	oral	0	0	0.71	0.14	-0.85		-0.12
R. F. 2 months	III	3.60	evap. milk + karo	oral	1.11	0.31	1.14	0.19	-0.22		-0.06
J. W. 3 weeks	III	3.12	glucose + saline evap. milk	i.v. oral	0 0.52	0 0.17	0.77	0.09	-0.34		-0.11

\* Abbreviations used are as follows: cas. hyd. = casein hydrolysate solution; c.h.f. = casein hydrolysate food. The composition is given in Footnote 3. i.v. = intravenous administration.

† These periods were irregular in length, varying from 1 to 4 days, and were consecutive. The Phases are numbered according to the type of therapy as follows: Phase I. Intravenous administration only. Phase II. Oral administration or intravenous and oral administration of casein hydrolysate solution. Phase III. Oral administration of casein hydrolysate food or milk formula instead of, or in addition to, casein hydrolysate solution (orally or intravenously). Blood transfusions were given sometimes in either Phase I, Phase II, or Phase III.

‡ Weight at beginning of period.

§ Estimated to allow for vomiting.

¶ Died.

basis of surface area, basal metabolism, or some other standard. There are 10 cases in which the same phase of treatment continued for 2 successive periods. In nearly all these cases, there was considerable difference in the nitrogen intake, due to blood transfusion or other variation. None were exact duplicates. In all cases, there was a change in the clinical status. For these reasons, the results are given separately rather than as averages.

It will be noted that the infants who received the casein hydrolysate (with or without blood transfusions) in adequate amounts, with one exception (E. P., first period), had positive balances. Even the 2 infants who were moribund (W. McG. and S. K.) showed good nitrogen retentions. On the other hand, the controls were in negative nitrogen balance. T. S. and J. T., who received glucose-saline solution only, had losses of the same order of magnitude as those previously reported for infants who had received no nitrogen, but liberal carbohydrate intakes (1, 3). R. F. and J. W., who were given milk formulas, also were in negative nitrogen balance. Their nitrogen intakes were low, but it was judged that no more could be tolerated because of the infants' clinical condition, which, moreover, was not as severe as that represented by the periods of observation recorded in Phase I for the other infants in this study.

The distribution of nitrogen in the excreta did not differ greatly from that found in normal babies. Even with infants who have diarrhea and are receiving intravenous therapy, fecal nitrogen is, in per cent of total output, no greater than in normal infants, 13.6 and 11.4 respectively (1). If the dry weight of the feces was high, the nitrogen excretion was high. The nitrogen forms a roughly constant proportion of from 2 to 7 per cent of the dry weight of the feces. This holds true of diarrheal stools and normal stools of infants fed parenterally and orally, whether given casein hydrolysate or milk. Furthermore, diarrheal stools do not show a great increase of nitrogen or of dry material; the main difference between them and normal stools lies in the water and salt content.

A brief summary of the data on nitrogen retention in Table I is given in Table II, arranged according to three levels of intake, irrespective of

TABLE II  
Average nitrogen balance with different levels of nitrogen intake, calculated from the data in Table I

Number of cases	Nitrogen intake		Nitrogen balance		
	Range	Average			
	grams per kgm. per day	grams per kgm. per day	grams per day	grams per kgm. per day	per cent of intake
6	0.27 to 0.35	0.31	-0.03	-0.01	
21	0.36 to 0.65	0.50	0.85	0.16	32
14	0.66 to 1.28	0.92	2.07	0.50	54

whether the nitrogen was ingested or injected. Retention is shown to be correlated with intake. With the amounts of nitrogen used in this study, when the intake is larger, the retention is larger also, but is not a constant fraction of the intake. A nitrogen intake greater than the amount of wastage is necessary to keep the individual in equilibrium. The average excretion of the controls, T. S. and J. T., was 0.19 gram per kgm. of body weight, whereas the amount of nitrogen necessary to maintain equilibrium is about 0.35 gram per kgm., which is equivalent to 2.8 grams of casein hydrolysate, or 2.2 grams of protein.

One case who received more than 0.35 gram of nitrogen had a small negative balance (E. P., first period), one was in equilibrium (J. J.), and 2 cases with intakes below this figure had small positive balances. Even in these sick children, positive balances were obtained with intakes as low as 0.29 (J. diB.) and 0.30 (R. C.). But 0.4 to 0.5 gram per kgm. of body weight is the minimum one would expect to use clinically.

There was only one case (E. P., first period) of negative balance when casein hydrolysate was given intravenously in sufficient amounts. The infant was in acidosis during this one-day period, the bicarbonate concentration of the plasma being 19 m.eq. per liter, which probably accounts for the extraordinarily high nitrogen excretion in the urine. Moreover, in the 3 day period immediately following, very high nitrogen retention was obtained.

In this series of cases, a number of blood transfusions were given. Presumably the plasma proteins thus introduced intravenously serve as an excellent source of nitrogen for nutrition (4). Even if they are not catabolized, but are used to build up depleted plasma proteins, it is still proper, in our opinion, to consider this nitrogen as re-

tained, for the same end result is reached directly, as that attained indirectly when proteins or amino acids are fed by mouth. In some cases, the transfusion supplemented an otherwise inadequate nitrogen intake, and in others, was added to a sufficient intake. For example, in the former case, the nitrogen balance would have been negative without the transfusion (D. D., second period), but in the latter, would have been positive without transfusion, and was rendered exceptionally high by the addition of the blood plasma (R. McK., second period).

The group with the highest nitrogen intake (see Table II) showed retentions which exceeded, both in amount and in proportion of intake, the retentions which may be expected in well infants. A normal infant on a milk formula diet usually retains about 25 per cent of the nitrogen ingested, shows a total retention of about 0.5 to 1.5 grams per day, or 0.17 gram per kgm. of body weight. This value, taken from our previous experience (1) agrees with the data in Czerny-Keller (5). The average proportion of intake retained by the sick infants in this study was 45 per cent, the highest 77 per cent (R. McK., fifth period); the highest actual retention was 4.0 grams per day (R. C., third period), and the highest retention per kgm. of body weight per day was 0.88 gram (R. McK., second period).

The high retentions may be attributed in part to larger intakes and in part to the fact that the nitrogen which had undergone previous depletion was now being restored. The previous loss from the tissues may have been due in part to lack of ability to digest or absorb protein, as well as to the withholding of food. It is easier to restore losses than to make new gains. Moreover, casein hydrolysate is more rapidly absorbed than unhydrolyzed protein (6).

Nitrogen retentions were not correlated with gains in weight. Although each gram of nitrogen retained carries with it approximately 6 times its weight of protein, or 30 times its weight of body substance, the body weight fluctuations depend primarily upon the state of hydration, rather than upon nitrogen accretion. One of the features of nutritional disturbances of this type is great loss of weight due to dehydration. It is therefore not uncommon for these cases to show astounding weight gains during recovery, of the

order of a kilogram or more in a few days. Some of this represents fluid replaced and some of it fluid which will subsequently be eliminated. These gains may be as much as 25 times the gain normally accompanying nitrogen retention of the order obtained in this study. The weights given in Table I were the weights at the beginning of each period, and the periods being of unequal length, they do not represent 24-hour gains.

Although increased body temperature has a tendency to increase protein catabolism, the effect in these cases was not marked. Although no attempt has been made to correlate nitrogen balance with temperature elevation, the loss of protein in the controls was not in excess of that of normal infants deprived of their nitrogen intake, and the positive balances of the cases studied were not less than might have been expected if no fever had been present.

Retentions were greater, both in amount and in proportion to the intake, when blood transfusions were given in conjunction with casein hydrolysate.

Whether the casein hydrolysate was administered intravenously, intravenously and orally, or orally, *i.e.*, whether the disease was in its most acute phase, a less acute phase, or the recovery phase, nitrogen retentions were approximately the same at the same levels of intake.

The present study shows that acute gastrointestinal disturbance does not hinder retention of nitrogen when administered in proper form and amount. The fact that these infants had good nitrogen balances when casein hydrolysate was given, but did not when saline-glucose solution or milk was given, is sufficient proof that casein hydrolysate administration is desirable. Other things being equal, the more nearly the normal physiological status can be approximated, the more adequate is the treatment. That it is better to prevent loss than to have to repair it, does not have to be shown by statistical treatment of clinical details.

#### SUMMARY

1. Infants with acute gastro-intestinal disturbance involving vomiting, diarrhea, dehydration, and acidosis, were able to retain nitrogen when it was given in adequate amounts in the form of casein hydrolysate, either intravenously or orally or both.

2. Positive nitrogen balances were obtained when the nitrogen intake was 0.35 gram per kgm. of body weight per day.

3. Retentions were greater when nitrogen intakes were greater, and were as large as those reported for well infants.

The author wishes to acknowledge the assistance of Dr. G. L. Brooks, who supervised the care of the infants; other members of the House Staff, who gave the intravenous injections; Miss Harriet O'Hara, who was the nurse in charge; and Miss Dorothy Cohen, who made the nitrogen determinations.

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# TRAUMATIC SHOCK. I. THE PRODUCTION OF RADIOACTIVE PLASMA PROTEIN FROM AMINO ACIDS CONTAINING RADIOACTIVE SULFUR

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In order to study the fate of the plasma proteins in shock, it was considered desirable to tag these proteins with radioactive elements. Sulfur was selected because of (1) its favorable half life (88 days), (2) its position of relative stability in the protein molecule, and (3) the significant role which sulfur-containing amino acids play in the generation of plasma proteins, as shown by Whipple *et al.* (1, 2). The preparation of radioactive sulfur-containing amino acids (cystine, methionine, and homocystine) from radioactive sulfur is reported by one of us in the following article in this same journal (3). This communication reports our experience in the production of radioactive plasma proteins by dogs fed radioactive sulfur-containing amino acids.

It has long been known that l-cystine is an amino acid essential for maintenance and growth of animals. Du Vigneaud *et al.* (4, 5), found that the l-cystine component of the meso form can be utilized by growing rats, but that d-cystine cannot be utilized. l-Cystine, necessary for the growth of rats, can be replaced by both d- and l-methionine (6) and by d- and l-homocystine (7) if choline or betaine are added to the diet (8). Tarver and Schmidt (9) isolated radioactive cystine from the fur of rats 3 weeks after feeding d,l-methionine containing radioactive sulfur.

Whipple *et al.* (1, 2) showed that hypoproteinemic dogs, which had been kept on a basal diet and depleted of "reserve stores" of plasma protein by long continued plasmapheresis, regenerated plasma protein at a markedly increased rate when l-cystine was fed, to a lesser extent when d,l-methionine was fed, and to a still lesser extent when l-tyrosine or l-tryptophane was fed. Homocystine was not studied. Whipple demonstrated that a hypoproteinemia of 4 grams per cent was the optimum level for maximum regeneration of plasma protein. A lower level of plasma pro-

tein did not permit maintenance of a satisfactory state of health.

With these facts as a basis, we studied the utilization of small amounts of radioactive d,l-cystine,<sup>1</sup> d,l-methionine, and d,l-homocystine by hypoproteinemic dogs, for the production of radioactive plasma protein.

## METHOD

Dogs were rendered hypoproteinemic after the manner described by Whipple (1), *i.e.* by the use of protein deficient diets and plasmapheresis. In most cases, however, the plasmapheresis was not carried on for the length of time required, according to Whipple, to deplete the "reserve stores" of plasma protein.

**Diet.** Dogs were fed a low protein diet for 2 weeks, followed by a non-protein diet for 1 week unless otherwise specified in the individual protocol. The diet was essentially that recommended by Cowgill (10). Each dog was given 70 to 80 calories per kilogram of body weight per day.

**Plasmapheresis** was performed aseptically 3 or 4 times weekly by removal of 25 to 30 per cent of the total blood volume (6 cc. of 5 per cent sodium citrate per 100 cc. blood as anticoagulant) with immediate return of the unwashed red cells in 2.5 per cent glucose in physiological saline solution.

Protein levels were determined by the specific gravity method. When a level of or close to 4 grams per cent was reached, an amount of plasma was removed during the following week which was just sufficient to maintain this level. The early death of some of the dogs is explained by the fact that much more plasma was removed with each plasmapheresis after the radioactive amino acids were given than before.

Amino acids containing radioactive sulfur were converted to the hydrochloride and then fed or, as in 2 cases, injected intravenously. Recorded weights are of free amino acids. At the same time, the diet was usually supplemented with 50 grams of casein daily (forced feeding when necessary). In most cases, l-tyrosine (1 gram)

<sup>1</sup> Although no utilization of d-cystine was expected, no attempt was made to resolve the radioactive d,l-cystine because of the small amounts available and the likelihood of some loss of the laevo form. A method for resolution of d,l-cystine is reported by du Vigneaud (4).



and in some cases 1-tryptophane (0.25 gram) was also fed daily. Dogs receiving homocystine were given betaine (4 grams) daily (8).

**Radioactivity determinations.** Because radioactive sulfur has a soft radiation, material containing it absorbs the radiation in proportion to the mass of the sample being measured. Because of this self-absorption, it is necessary to utilize the thinnest possible layer for measurement of the radioactivity. The most convenient form in which to obtain the sulfur from biological material for measurement is by oxidation of the material and precipitation of the sulfur as either barium or benzidine sulfate. As recommended by Tarver and Schmidt (9), the barium sulfate layer should not exceed 3 mgm. per sq. cm.

Plasma or tissue was prepared for analysis of radioactive potency by oxidation of 1 cc. of plasma or 1 gram of tissue with concentrated nitric acid and superoxol in a 100 cc. Kjeldahl flask. This required about 1.5 to 2.0 hours. Care was taken to avoid ignition when the mixture approached dryness. The non-protein fraction of plasma was similarly prepared for analysis after removal of the protein fraction by precipitation with an equal volume of 20 per cent trichloroacetic acid. The resulting ash in both cases was dissolved in several cc. of water containing 1 or 2 drops of concentrated hydrochloric acid.

When isolation of the sulfur as  $\text{BaSO}_4$  was desired, the solution was transferred to a large test tube, diluted to 10 cc. and heated to boiling. An equal volume of 0.1 per cent  $\text{BaCl}_2$  solution brought to the boiling point was added to the hot sulfate solution and the mixture was digested for 1 hour and allowed to stand overnight. It was again digested for 1 hour at  $100^\circ \text{C}$ ., and the precipitate was collected while hot.

When isolation of the sulfur as benzidine sulfate was desired, the solution was transferred to a 50 cc. beaker, evaporated to 3 cc., and diluted with an equal volume of alcohol. To this was added 1 cc. of a saturated solution of benzidine dihydrochloride in 50 per cent alcohol, containing a few drops of concentrated hydrochloric acid. The mixture was allowed to sit in the ice box half an hour and the precipitate was collected.

Either precipitate was collected in a filtration apparatus, similar to the one described by Tarver and Schmidt (9) except that the precipitate covered a 1.54 sq. cm. area. It was washed with 50 per cent alcohol, water, and finally with acetone.

Advantages of the benzidine method are (1) the precipitates (7 mgm.) are easier to collect than the barium

precipitates (4.5 mgm.), (2) the particle size is more uniform, and (3) the procedure can be carried out more quickly and efficiently.

The weight of the precipitate was determined in every case. Since analyses of urine and feces were made on aliquots treated in the same way as plasma, care was taken to select the size of the aliquot that would yield approximately the same weight of sulfate precipitate.<sup>2</sup>

Standards were prepared by oxidizing a known weight of the radioactive amino acid fed, and dissolving the ash in a given volume of  $\text{Na}_2\text{SO}_4$  solution which was equivalent to 4.5 mgm.  $\text{BaSO}_4$  per cc. Several dilutions of the radioactive solution were made with this non-radioactive  $\text{Na}_2\text{SO}_4$  solution. In this way, standard precipitates of the same weight, containing known dilutions of the original radioactive amino acid, were obtained (Table I).

TABLE I  
*Radioactivity measurements of the standards  
in one experiment*

Dilutions of radioactive standard	Cystine equivalent	Weight of benzidine sulfate	Radioactivity	Ratio of radioactivity to background	Deviation from linearity
	mgm.	mgm.	divisions per second		per cent
1	0.80	10.5	0.033	250	-13
25	0.032	9.7	0.0015	10	-1.3
100	0.008	9.5	0.00038	3	0
1000	0.0008	9.3	0.00028	0.2	-26

All measurements were made with a modified Lauritzen electroscope, described in another publication (11). In subsequent protocols, radioactivity is expressed in divisions per second (each division equals 20 small divisions on the scale of the electroscope). The background of our instrument varied from 0.00006 to 0.00009 division per second.

In the experiments which follow, the total amount of radioactive plasma protein removed from the dog is given as percentage of the amino acid fed. By so doing, a basis is provided for estimating the radioactive potency of the amino acids necessary to obtain any desired level of radioactivity in the plasma protein.

<sup>2</sup> We found a rough practical method of doing this was to use  $\frac{1}{400}$  to  $\frac{1}{600}$  of a 24-hour output.

## EXPERIMENTS WITH RADIOACTIVE CYSTINE, HOMOCYSTINE, AND METHIONINE

TABLE II

*Radioactive cystine*

Dog No. S-11; weight 6.8 kgm.; hematocrit 40 per cent; non-protein diet 6 days, low protein diet 6 days; plasmapheresis 5 times; lowest plasma protein level 4.5 grams per cent; weight loss 1 kgm.; radioactive d,1-cystine (200 mgm.) fed; low protein diet supplemented with casein (50 grams) and 1-tyrosine (1 gram) daily; circulatory collapse after third blood withdrawal; restored with saline and plasma; dog decorticate until termination of experiment 2 days later; weight 5 kgm.; hematocrit 25 per cent.

Specimen	Duration of experiment	Total protein	Radio-active plasma removed	Radioactivity per cc. plasma			Radioactive cystine in plasma protein		Ingested radio-active 1-cystine (100 mgm.) utilized
				Plasma	Non-protein fraction	Protein fraction	Per cc. plasma	Total plasma removed	
	<i>days</i>	<i>per cent</i>	<i>cc.</i>	<i>divisions per second</i>			<i>mgm.</i>	<i>mgm.</i>	<i>per cent</i>
Plasma	0	4.5							
Plasma	1	5.1	20	0.00153	0.00043	0.00110	0.024	0.48	
Plasma	2	5.5	15	0.00109	0.00018	0.00091	0.020	0.30	
Plasma	3	5.5	150	0.00089	0.00008	0.00081	0.018	2.70	
Plasma	5	6.8	175	0.00045		0.00045	0.010	1.75	
						Total	5.23		5.2
			Volume	Radioactivity per cc.	Radioactive cystine				
					Per cc.	Total specimen			
Urine	2		<i>cc.</i>	<i>divisions per second</i>	<i>mgm.</i>	<i>mgm.</i>		36* 1.2*	
Feces	2		400 250	0.00807 0.00044	0.18 0.0099	72† 2.5			
			Weight organ	Radioactivity per gram	Radioactive cystine				
					Per gram	Total organ			
			<i>grams</i>	<i>divisions per second</i>	<i>mgm.</i>	<i>mgm.</i>			
Liver	5		250	0.00090	0.020	5.0			
Lung	5		63	0.00097	0.021	1.3			
Kidney	5		50	0.00096	0.021	1.0			
Bowel	5		200	0.00090	0.020	4.0			
Heart	5		42	0.00068	0.015	0.6			
Brain	5		50	0.00012	0.003	0.2			
Leg muscle	5		2000	0.00030	0.007	14.0			

Standard from 0.0080 mgm. cystine had activity of 0.00036 divisions per second.

\* Calculated on basis of d,1-cystine ingested.

† Excreted as sulfate.

TABLE III

*Radioactive cystine*

Dog No. S-19; weight 9.1 kgm.; low protein diet 22 days, non-protein diet 9 days; plasmapheresis 16 times; lowest plasma protein level 5.8 grams per cent; weight loss 1.3 kgm.; radioactive d,1-cystine (400 mgm.) fed in 2 doses at 24-hour interval; non-protein diet supplemented with casein (50 grams), 1-tyrosine (1 gram), and 1-tryptophane (0.25 gram) daily; dog died 1 week after last sample withdrawn.

Specimen	Duration of experiment	Total protein	Radio-active plasma removed	Radioactivity per cc. plasma			Radioactive cystine in plasma protein		Ingested radio-active 1-cystine (100 mgm.) utilized
				Plasma	Non-protein fraction	Protein fraction	Per cc. plasma	Total plasma removed	
	<i>days</i>	<i>per cent</i>	<i>cc.</i>	<i>divisions per second</i>			<i>mgm.</i>	<i>mgm.</i>	<i>per cent</i>
Plasma	0	5.8							
Plasma	1	5.1	164	0.00220	0.00124	0.00096	0.020	3.30	
Plasma	2	5.5	152	0.00301	0.00105	0.00196	0.040	6.10	
Plasma	3	5.5	134	0.00278	0.00052	0.00226	0.046	6.20	
Plasma	4	5.8	150	0.00260	0.00072	0.00188	0.038	5.70	
Plasma	5	5.1	191	0.00178	0.00041	0.00137	0.028	5.30	
Plasma	8	6.2	172	0.00118	0.00027	0.00091	0.019	3.25	
						Total	29.85		14.8
			Volume	Radioactivity per cc.	Radioactive cystine				d, 1-Cystine
					Per cc.	Total specimen			
Urine	3		<i>cc.</i> 500	<i>divisions per second</i> 0.00728	<i>mgm.</i> 0.15	<i>mgm.</i> 75*			<i>per cent</i> 19

Standard from 0.0080 mgm. cystine had activity of 0.00039 divisions per second.

\* Excreted as sulfate.

TABLE IV

*Radioactive cystine*

Dog No. S-18; weight 14.5 kgm.; non-protein diet 5 days; plasmapheresis once; radioactive d,1-cystine (174 mgm.) fed; non-protein diet supplemented with casein (50 grams), 1-tyrosine (1 gram), and 1-tryptophane (0.25 gram) daily; dog died 3 days later with bronchopneumonia.

Specimen	Duration of experiment	Total protein	Radio-active plasma removed	Radioactivity per cc. plasma			Radioactive cystine in plasma protein		Ingested radioactive 1-cystine (87 mgm.) utilized
				Plasma	Non-protein fraction	Protein fraction	Per cc. plasma	Total plasma removed	
	<i>days</i>	<i>per cent</i>	<i>cc.</i>	<i>divisions per second</i>			<i>mgm.</i>	<i>mgm.</i>	<i>per cent</i>
Plasma	0	7.2							
Plasma	1	6.8	118	0.000529	0.000272	0.000257	0.0068	0.80	
Plasma	2	5.2	130	0.000339	0.000107	0.000232	0.0062	0.81	
Blood	3		5	0.000122					
						Total	1.61		1.84
			Volume	Radioactivity per cc.	Radioactive cystine				d,1-Cystine
					Per cc.	Total specimen			
Urine	3		<i>cc.</i> 1000	<i>divisions per second</i> 0.00219	<i>mgm.</i> 0.058	<i>mgm.</i> 58*			<i>per cent</i> 34

Standard from 0.0080 mgm. cystine had activity of 0.00030 divisions per second.

\* Excreted as sulfate.

# PRODUCTION OF RADIOACTIVE PLASMA PROTEIN

TABLE V

## Radioactive homocystine

Dog No. S-24; weight 8.4 kgm.; low protein diet 10 days, non-protein diet 19 days; plasmapheresis 10 times; lowest plasma protein level 4.1 grams per cent; radioactive d,l-homocystine (500 mgm.) fed; non-protein diet supplemented with gelatin (30 grams) and betaine hydrochloride (4 grams) daily; dog died suddenly 5 days later; weight 7.1 kgm.

Radioactive homocystine										
Specimen	Duration of experiment	Total protein	Radioactive plasma removed	Radioactivity per cc. plasma			Radioactive cystine in plasma protein		Ingested radioactive d,l-homocystine (500 mgm.) utilized	
				Plasma	Non-protein fraction	Protein fraction	Per cc. plasma	Total plasma removed		
Plasma	days	per cent	cc.	divisions per second			mgm.	mgm.	per cent	
Plasma	0	4.1	15	0.00030	0.00028	0.00005	0.0016	0.24		
Plasma	1	3.9	150	0.00033						
Plasma	2	4.2	15	0.00014						
Plasma	3	3.8	15	0.00011						
Plasma	4	4.1	150	0.00011						
Plasma	5	4.3								
					0.00001	0.00010	0.0032	0.05		
					0.00001	0.00010	0.0032	0.49		
						Total		0.78		
						Radioactive homocystine			0.15	
			Volume	Radioactivity per cc.		Per cc.		Total specimen		
			cc.	divisions per second		mgm.	mgm.			
Urine	2		200	0.0250		1.55	310*		62.0	
Urine	3		200	0.0013		0.08	16*		3.2	
Urine	4		200	0.0005		0.017	3.3*		0.7	
Feces	2		50	0.0032		0.19	9.8		2.0	
						Total				
			Weight of organ	Radioactivity per 1.9 grams		Radioactive homocystine			68	
			grams	divisions per second		Per 1.9 grams	Total organ			
Liver	5		336	0.00034		mgm.	mgm.			
Lung	5		59	0.00026		0.0110	1.9		0.4	
Skin	5		920	0.00018		0.0084	0.26		0.05	
Muscle	5		2400	0.00012		0.0059	2.8		0.6	
						0.0039	4.9		1.0	

Standard from 0.102 mgm. homocystine had activity of 0.00166 divisions per second.  
Standard from 0.026 mgm. homocystine had activity of 0.00080 divisions per second.

\* Excreted as sulfate.

Standard from 0.102 mgm. homocystine had activity of 0.00166 divisions per second.  
Standard from 0.026 mgm. homocystine had activity of 0.00080 divisions per second.

\* Excreted as sulfate.

TABLE VI

*Radioactive homocystine*

Dog No. S-25; weight 8.4 kgm.; hematocrit 45 per cent; low protein diet 17 days; plasmapheresis 10 times; lowest plasma protein level 3.8 grams per cent; hematocrit 20 per cent; radioactive d,l-homocystine (50 mgm.) intravenously; low protein diet supplemented with casein hydrolysate (50 grams) daily; dog died 6 days later with bronchopneumonia, pulmonary edema, liver necrosis, and edema.

Specimen	Duration of experiment	Total protein	Radioactive plasma removed	Radioactivity per cc. plasma			Radioactive homocystine in plasma protein		Injected radioactive d,l-homocystine (50 mgm.) utilized
				Plasma	Non-protein fraction	Protein fraction	Per cc. plasma	Total plasma removed	
	<i>days</i>	<i>per cent</i>	<i>cc.</i>	<i>divisions per second</i>			<i>mgm.</i>	<i>mgm.</i>	<i>per cent</i>
Plasma	0	3.8							
Plasma	1		10	0.00007					
Plasma	2	4.1	150	0.00003	0.00001	0.00002	.0031	0.47	0.9
Plasma	3	3.9	150	0	0	0	0	0	0
			Volume		Radioactivity per cc.	Radioactive homocystine			
						Per cc.	Total specimen		
			<i>cc.</i>	<i>divisions per second</i>		<i>mgm.</i>	<i>mgm.</i>		
Urine	2		200	0.00011		0.017	3.4*		6.8
Urine	5		200	0		0	0*		0

Standard from 0.025 mgm. homocystine had activity of 0.00016 divisions per second.

\* Excreted as sulfate.

TABLE VII

*Radioactive methionine*

Dog No. S-26; weight 9 kgm.; non-protein diet 15 days; plasmapheresis twice; lowest plasma protein level 4.1 grams per cent; radioactive d,l-methionine (50 mgm.) fed; non-protein diet supplemented with casein hydrolysate (50 grams) daily, and non-radioactive l-cystine (50 mgm.) twice; experiment terminated after 11 days.

Specimen	Duration of experiment	Total protein	Radioactive plasma removed	Radioactivity per cc. plasma		Radioactive methionine in plasma protein		Ingested radioactive d,l-methionine (50 mgm.) utilized
				Plasma	Protein* fraction	Per cc. plasma	Total plasma removed	
	<i>days</i>	<i>per cent</i>	<i>cc.</i>	<i>divisions per second</i>		<i>mgm.</i>	<i>mgm.</i>	<i>per cent</i>
Plasma	0	4.1						
Plasma	1	3.8	15	0.000057				
Plasma	2	3.6	150	0.000036	0.000005	0.00017	0.025	
Plasma	6	3.7	15	0.000029				
Plasma	11	4.6	15	0.000010	0.000010	0.00034	0.005	
						Total	0.03	0.06
			Volume	Radioactivity per cc.		Radioactive methionine		
						Per cc.	Total specimen	
			<i>cc.</i>	<i>divisions per second</i>		<i>mgm.</i>	<i>mgm.</i>	
Urine	2		400	0.00058		0.02	8†	16
Urine	4		200	0.00014		0.005	1†	2
Feces	4		100	0.00069		0.024	2.4	5

Standard from 0.0028 mgm. methionine had activity of 0.000082 divisions per second.  
Standard from 0.028 mgm. methionine had activity of 0.00081 divisions per second.

\* Determination of radioactivity made on dialysed specimen.

† Excreted as sulfate.

TABLE VIII

*Radioactive methionine*

Dog No. S-27; weight 8.0 kgm; hematocrit 45 per cent; non-protein diet 16 days; low protein diet 10 days; non-protein diet 26 days; plasmapheresis 10 times; lowest plasma protein level 4.0 grams per cent; weight loss 1.2 kgm; radioactive d,1-methionine (150 mgm.) intravenously; non-protein diet supplemented with gelatin (50 grams) and 1-tyrosine (1 gram) daily; experiment terminated after 7 days; hematocrit 24 per cent.

Specimen	Duration of experiment	Total protein	Radioactive plasma removed	Radioactivity per cc. plasma		Injected radioactive d,1-methionine (150 mgm.) utilized
				Plasma	Protein fraction	
Plasma	days	per cent	cc.	divisions per second		per cent
Plasma	0	4.0	6	0.000035	0	0
Plasma	1	4.1	15	0.000018	0	
Plasma	2	4.6	30	0.000035	0	
Plasma	7	4.6	15	0	0	
Urine Urine Urine Feces	2 4 7 7	Volume cc. 400 300 600 200	Radioactivity per cc. divisions per second 0.00070 0.000065 0.000021 0.000012	Radioactive methionine		11 1.6 1.2 0.1
				Per cc.	Total specimen	
				mgm.	mgm.	
				0.040 0.008 0.003 0.001	16* 2.4* 1.8* 0.2	

Standard from 0.0029 mgm. methionine had activity of 0.000024 divisions per second.  
Standard from 0.029 mgm. methionine had activity of 0.00052 divisions per second.

\* Excreted in sulfate.

TABLE IX

*Summary of the results of the individual experiments*

Dog number	Table number	Amino acid	Weight fed	Total plasma removed	Highest concn. of amino acid in protein fraction of plasma	Ingested amino acid		Diet supplements and remarks
						Incorporated in plasma protein	Excreted in urine as sulfate	
			mgm.	cc.	mgm. per cc.	per cent	per cent	
I	II	1-Cystine	100	360	0.024	5.2	36†	Casein, tyrosine
II	III	1-Cystine	200	963	0.046	14.8	19†	Casein, tyrosine, tryptophane
III	V	d,1-Homocystine	500	345	0.0032	0.15	66	Gelatin, betaine
IV	VI	d,1-Homocystine	50*	310	0.0031	0.9	7	Casein hydrolysate, infection
V	VII	d,1-Methionine	50	195	0.00034	0.06	18	Casein hydrolysate, 1-cystine
VI	VIII	d,1-Methionine	150*	66	0.0000	0.00	14	Gelatin, 1-tyrosine

\* Injected intravenously.

† Calculated on basis of d,1-cystine.

## RESULTS

A summary of the results of the individual experiments is given in Table IX. The most efficient utilization of sulfur-containing radioactive amino acids in the production of plasma protein was obtained from cystine. Calculations were made on the basis of the 1-cystine content of the ingested d,l-cystine, since d-cystine is not utilized. A comparison of dog 11 and dog 19 (Tables II and III) shows that the ingestion of 2 doses of cystine at a 24-hour interval resulted in a concentration of cystine in plasma protein twice that obtained with one dose. Maximum levels were obtained in 24 hours. However, the total percentage utilization, which is calculated on the basis of the amount of plasma which can be removed without injury, was 3 times as great. This result may have been due to the fact that dog 19 was an animal in better condition than dog 11. That dog 19 was a superior plasma producer was evident from the fact that repeated plasmapheresis reduced the plasma protein to 5.8 grams per cent slowly.

The importance of hypoproteinemia as a stimulus to plasma protein production and to the incorporation of cystine in the plasma proteins is suggested by results from dog 18 (Table IV). The plasma total protein was 7.2 grams per cent (plasmapheresis for induction of hypoproteinemia was not done) at the time radioactive cystine was fed. Only 1.8 per cent of the 1-cystine fed was incorporated in the plasma protein withdrawn, in contrast to 5 per cent and 15 per cent in dogs 11 and 19. But since dog 18 developed a fatal infection, the latter may also have been an important factor in producing poor utilization of cystine.

Much poorer utilization was obtained with homocystine and methionine than with cystine. This confirms Whipple's observations on the relative importance of methionine and cystine in plasma protein production by hypoproteinemic dogs kept on a low protein basal diet (2). Supplementary betaine did not improve the utilization of small amounts of homocystine (cf. dogs 24 and 25 (Tables V and VI)). In comparing dogs 26 and 27 (Tables VII and VIII), inactive cystine may have exerted a slightly beneficial effect, but tyrosine exerted none. No evidence was obtained in the experiments with homocystine

and methionine that intravenous administration of the amino acids increased their utilization in the production of plasma protein.

The concentration of radioactive cystine in liver, lung, kidney, and bowel were found to be about the same in one experiment. Less was found in muscle, and much less in brain. Similar data are given for homocystine in one experiment.

## SUMMARY

Dogs made hypoproteinemic, according to Whipple's method (1) of low protein intake and plasmapheresis, were fed cystine, homocystine, or methionine containing radioactive sulfur. Incorporation of these amino acids in the plasma protein was obtained in each case. The highest concentration of amino acid in the plasma protein was obtained with cystine. The largest percentage utilization of amino acid in the production of radioactive plasma protein was obtained with cystine.

Data are provided in Table IX for estimating the radioactive potency of the amino acids necessary to obtain any desired level of radioactivity in the plasma protein.

## CONCLUSION

A technique for the preparation of plasma protein tagged with radioactive sulfur, by the utilization of dogs made hypoproteinemic according to Whipple's technique and fed radioactive amino acids, has been developed. From dogs so prepared, the plasma, withdrawn and dialysed, provides a source of radioactive sulfur-containing plasma proteins in sufficient quantity to make possible their use in any study involving the movement of plasma protein into or out of the circulation.

Acknowledgment is due Mr. Alexander M. Rutenburg and Miss Martha Goldberg for technical assistance.

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# TRAUMATIC SHOCK. II. THE PREPARATION OF CYSTINE, METHIONINE, AND HOMOCYSTINE CONTAINING RADIOACTIVE SULFUR

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In order to prepare, from radioactive sulfur, the sulfur-containing amino acids of a high order of specific activity for biological experiments such as those described in the foregoing publication, it was necessary (owing to the cost of radioactive sulfur) to investigate the efficiency of utilization of small amounts of sulfur. The synthetic methods utilized are not novel, but are described below because of the data obtained on yields in numerous small scale preparations. Since present methods of preparing radioactive sulfur from neutron bombardment of carbon tetrachloride involve its isolation and purification as  $\text{BaSO}_4$  (1), the schema of synthesis used for the preparation of three sulfur containing amino acids begins with barium sulfate (Figure 1).

The synthesis of radioactive methionine (VI) from radioactive sulfur (0.05 moles) was reported by Tarver and Schmidt (2), using a modification of the synthesis of homocystine (V) reported by Patterson and du Vigneaud (3). The same yields as they report were obtained in preparing homocystine from 0.06 moles of sulfur. However, in the methylation of homocysteine, we were unable to obtain the anticipated yields. It was found that for preparing homocystine, the more convenient reduction of benzylhomocysteine (IV) with sodium and butyl alcohol gave as good yields as reduction with sodium and liquid ammonia. For the preparation of methionine, treatment of the resultant sodium salt of homocysteine with methyl iodide did not give as good yields as reported by Tarver and Schmidt, whether reduction was conducted in liquid ammonia or butyl alcohol. Attempted methylation with dimethyl sulfate and diazomethane did not proceed well.

The method of synthesis of cystine (IX) from

benzyl mercaptan (I) (0.6 moles), reported by Wood and du Vigneaud (4) in 23 per cent yield, was found to give a 21.5 per cent yield when 0.06 mole was used. Reduction of benzylcysteine (VIII) to cysteine with sodium and butyl alcohol did not give good yields; therefore, sodium and liquid ammonia were used. Since radioactive benzyl mercaptan is necessary for the synthesis of all three amino acids, a method of preparation of the mercaptan from hydrogen sulfide, other than that described by Tarver and Schmidt, in 70 per cent yield, was investigated. Elementary sulfur was prepared from hydrogen sulfide by oxidation with iodine, and this was treated with benzylmagnesium chloride. The yield of benzyl mercaptan was 80 per cent. It was found that the yield by the method of Tarver and Schmidt could be raised 4 per cent if the small amount of dibenzylsulfide formed in their reaction was reduced to benzyl mercaptan with sodium in liquid ammonia.

The Fischer synthesis (5) of cystine from  $\beta$ -chloroaminopropionic acid with barium hydro-sulfide was investigated under various conditions. Much poorer yields than the 5 to 10 per cent reported on the basis of chloroaminopropionic acid were obtained when sulfur was not used in large excess.

Attempts to prepare radioactive cystine by replacement reactions of cysteine and radioactive hydrogen sulfide have been described by Tuck (6) and Tarver and Schmidt (2). In a reinvestigation of the problem, no more than 0.3 per cent of available radioactivity was incorporated in cystine (two crystallizations) obtained after heating cysteine hydrochloride with radioactive hydrogen sulfide in aqueous solution at 105° C. for 10 hours. Heating for longer intervals, at higher temperatures, or at alkaline pH, resulted in destruction of a large fraction of the cysteine.

The enzymatic preparation of cysteine from radioactive hydrogen sulfide has been reported by

<sup>1</sup> Part of this work was done in partial fulfillment of the Bachelor's Degree with honors in biochemistry, Harvard University.

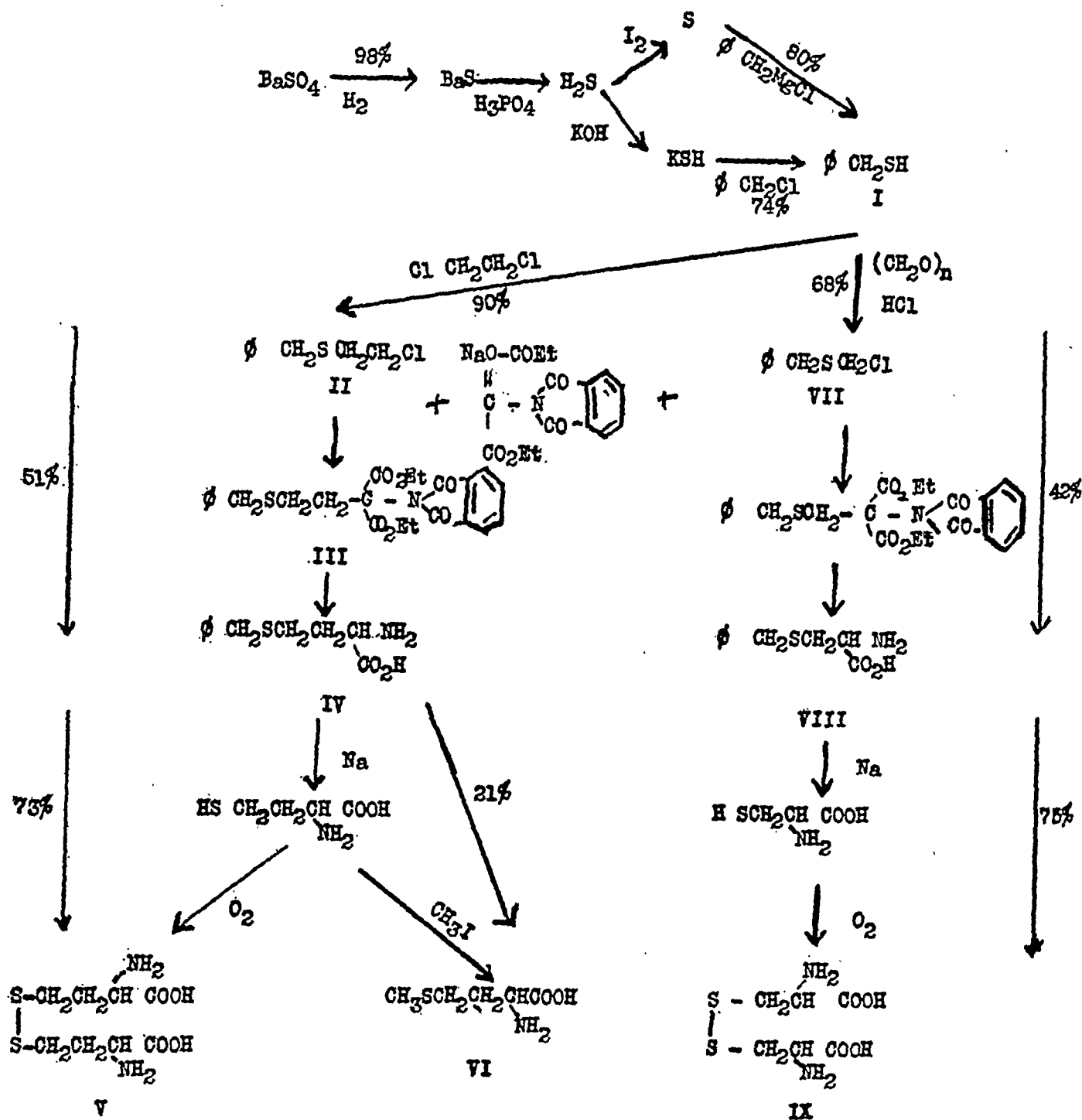


FIG. 1. SCHEME FOR SYNTHESIS OF CYSTINE, METHIONINE, AND HOMOCYSTINE FROM RADIOACTIVE SULFUR

Smythe and Halliday (7). The isolation of glutathione, from yeast grown on media containing radioactive magnesium sulfate, has been reported by Franklin (8).

#### EXPERIMENTAL

Reduction of  $\text{BaSO}_4$  was accomplished by passing  $\text{H}_2$  over finely ground barium sulfate at  $750^\circ \text{C}$ . The rate of hydrogen flow was determined by bubbling the gas through concentrated sulfuric acid. A Vicor glass tube, 1 inch in diameter, was heated by means of an encircling

electric oven 15 inches in length. The temperature was determined once by means of a thermocouple. Escaping gas was bubbled through ammoniacal cadmium sulfate solution, protected by a trap to eliminate the danger of sucking back. The barium sulfate was spread over the bottom of a porcelain boat, avoiding a layer more than 3 mm. in depth. In a characteristic run, 3.14 grams of barium sulfate were reduced for 5 hours. The loss of weight due to water formed amounted to 85 per cent of the theoretical amount. The sintered barium sulfide was ground to a powder and the reduction continued for another 3 hours. The additional water loss amounted to 5 per cent of the theoretical amount, and the cadmium sul-

fide formed from the escaping gas amounted to 7.8 per cent; total yield of both sulfides, 97.8 per cent. The oxidation and hydrolysis of barium sulfide was prevented by sealing under glass.

Hydrogen sulfide production occurred more rapidly at the beginning of reduction when water was seen condensing in the cold part of the tube.

**Sulfur.** Hydrogen sulfide was generated from barium sulfide with 30 per cent phosphoric acid and from cadmium sulfide with concentrated hydrochloric acid. The gas was swept out of the reaction flask with nitrogen and bubbled through two absorption tubes, containing slightly more than an equivalent of iodine dissolved in 20 per cent potassium iodide. The excess iodine was reduced with a little sodium bisulfite and the mixture was neutralized with ammonium hydroxide. The mixture was then heated at 100° C. for 1 hour to convert the amorphous sulfur to the crystalline form. It was ground to a powder, collected, and washed with water, alcohol, and ether. The yield was quantitative.

**Benzyl mercaptan (I) from sulfur.** Benzyl magnesium chloride, prepared from 0.12 mole of benzyl chloride and 0.13 mole of magnesium, was allowed to remain in the presence of the excess magnesium overnight to ensure complete reaction of the benzyl chloride. To this was added 2.1 grams (0.065 mole) of sulfur. The mixture was agitated for 24 hours until the sulfur had disappeared. The reaction product was then decomposed with ice and ammonium chloride, extracted with ether, washed, dried, and distilled. Benzyl mercaptan (6.5 grams, 80 per cent yield) distilled as a colorless liquid (b.p. 194°). The residue, 2.2 grams (b.p. 2 mm. 130° C.), after reduction with sodium in liquid ammonia failed to give further mercaptan.

**Benzyl mercaptan (I) from hydrogen sulfide.** Hydrogen sulfide, passed over a small amount of anhydrous calcium chloride, was absorbed in an equivalent of cold 2 N alcoholic potassium hydroxide (0.065 mole). An equivalent of benzyl chloride dissolved in 2 volumes of dry ether was added at once. A fluffy precipitate of potassium hydrosulfide formed, and was gradually replaced by a granular precipitate of potassium chloride on standing in the cold for 15 minutes. The mixture was then heated to the boiling point, after taking the precaution to absorb the hydrogen sulfide which escaped, an additional 2 N alcoholic potassium hydroxide (10 per cent of the total). This was treated with an additional 10 per cent of the benzyl chloride used, added to the reaction mixture, and allowed to sit several hours. After evaporation of most of the ether, the mixture was used directly in the preparation of benzylthioethyl chloride (II). For the preparation of benzylthiomethyl chloride (III), benzyl mercaptan was isolated. Distillation of the filtrate gave 6 grams (74 per cent yield) of benzyl mercaptan (b.p. 194°), and 1.5 grams (4.6 per cent yield) of dibenzyl sulfide. Reduction of the latter with sodium and liquid ammonia gave 0.3 gram (4 per cent yield) of benzyl mercaptan; total yield 6.3 grams (78 per cent). Tarver and Schmidt did not isolate dibenzyl sulfide, and

reported a yield of 70 per cent on the basis of the titration of an aliquot.

#### SYNTHESIS OF HOMOCYSTINE

**Benzylthioethyl chloride (II).** To the concentrated reaction mixture containing benzyl mercaptan in 74 per cent yield as described above, was added an equivalent of sodium methylate in the cold. Ten moles of ethylene dichloride (50 grams) were added and the mixture was allowed to stand overnight. It was then heated to the boiling point, cooled, acidified, and washed with water. The washings were extracted with a small amount of ethylene chloride. The combined organic layer was dried with anhydrous sodium sulfate, and the ethylene dichloride distilled at atmospheric pressure. The pressure was lowered and the fraction boiling at 100 to 110° C. at 3 mm. was taken. The yield was 8 grams (90 per cent) of pale yellow oil.

**Benzylthioethyl phthalimidomalonate (III).** The above oil was heated with 18 grams of sodium phthalimidomalonate (9), at 170° C. for 5 hours. The product was obtained by extraction with toluene.

**S-Benzylhomocysteine (IV).** The oily product was refluxed with 20 cc. alcohol and 35 cc. 5 N NaOH for 30 minutes. Water (100 cc.) and 20 cc. concentrated HCl were added and refluxing was continued for 30 minutes. After the addition of 50 cc. concentrated HCl, refluxing was continued for 1 hour. The solution was then cooled and extracted with ether, and the aqueous portion was evaporated to dryness at reduced pressure. The residue was extracted with boiling absolute alcohol and neutralized with  $\text{NH}_4\text{OH}$ . The precipitate was collected, washed with hot alcohol, and ether. The yield of fine white platelets was 5 grams (51 per cent). On the basis of  $\text{BaSO}_4$ , utilizing a 74 per cent yield of benzyl mercaptan, the yield was 33 per cent; that reported by Tarver and Schmidt was 29 per cent.

**dl-Homocystine (V).** Benzylhomocysteine (1.56 grams) was suspended in 30 cc. butyl alcohol, and 1.6 grams of sodium were added in small pieces to the refluxing mixture over 2.5 hours. After cooling, the solution was extracted with water, the aqueous extract was neutralized with HCl and made faintly alkaline with  $\text{NH}_4\text{OH}$ . A crystal of  $\text{FeCl}_3$  was added, and  $\text{O}_2$  was bubbled

into the solution until the color of the dark complex which formed had disappeared. The solution was neutralized, concentrated *in vacuo* to 75 cc., and allowed to stand overnight. The precipitate was collected, washed with water, alcohol, and ether. The mother liquor was concentrated and yielded additional homocystine; total yield 0.6 gram (73 per cent) of fine white crystals. The yield based on  $\text{BaSO}_4$  was 24 per cent.

#### SYNTHESIS OF METHIONINE

*dl-Methionine (VI)*. Benzylhomocysteine was reduced with sodium and butyl alcohol, as described for the preparation of homocystine. The solution was cooled to  $-10^\circ\text{C}$ . Seven molecular equivalents of methyl iodide were added, and the solution was kept at that temperature for 30 minutes. Water was added; the solution was neutralized and concentrated *in vacuo* to a small volume. After standing, methionine (as white leaflets) was collected in 21 per cent yield. When more methyl iodide or less was used, and the reaction conducted at a higher temperature, less methionine was recovered. The yield was not improved by following the procedure of Tarver and Schmidt.

The yield on the basis of sulfur used, reported by Tarver and Schmidt, was 19.5 per cent.

#### SYNTHESIS OF CYSTINE

*Benzylthiomethyl chloride (VII)*. Following the procedure of Wood and du Vigneaud, using 6.3 grams of benzyl mercaptan, 2.1 grams of polyoxymethylene, 2.5 grams of  $\text{CaCl}_2$  and dry hydrogen chloride, 6 grams (68 per cent yield) benzylthiomethyl chloride was obtained after distillation at water pump pressure (b.p.  $130^\circ$ ). A crystalline residue boiling about  $200^\circ\text{C}$ . weighed 1.9 grams, probably consisting of di(benzylthio)methane. This same product was obtained in high yield when sodium benzyl mercaptide was treated with an excess of methylene dichloride.

*S-Benzylcysteine (VIII)*. A mixture of 6 grams of benzylthiomethyl chloride, 14 grams of sodium phthalimidomalonate ester, and 30 cc. of toluene was refluxed for  $2\frac{1}{2}$  hours, according to the procedure of Wood and du Vigneaud. After filtration and evaporation of the toluene, the viscous oil was hydrolysed according to their pro-

cedure. After washing with hot alcohol, the yield was 3.1 grams (42 per cent) of fine white platelets.

*dl-Cystine (IX)*. The reduction of 1.25 grams of benzylcysteine with sodium in liquid ammonia and oxidation to cystine, according to the procedure of Wood and du Vigneaud, gave 0.53 grams (75 per cent yield). Overall yield on the basis of  $\text{BaSO}_4$  was 17 per cent. The yield from benzyl mercaptan was 21.5 per cent; Wood and du Vigneaud obtained a 23 per cent yield. The reduction of benzyl cysteine with sodium and butyl alcohol, as already stated, yielded only traces of cystine.

#### FISCHER SYNTHESIS

Bombs containing  $\beta$ -chloroaminopropionic acid (10) were heated at  $100^\circ\text{C}$ . for several hours with an equivalent of  $\text{Ba}(\text{SH})_2$ , KSH, and benzyl mercaptan in alcoholic, aqueous and dioxane medium. In no case was cystine or benzylcysteine isolated. The chloroaminopropionic acid was decomposed in each case and the odor of acrylic acid was noted when the tubes were opened.

#### SULFUR EXCHANGE EXPERIMENT

In preliminary experiments it was found that cysteine hydrochloride largely decomposed when heated with more than an equivalent of potassium hydrosulfide in a bomb at  $100^\circ\text{C}$ . When sufficient hydrochloric acid was added to the cysteine hydrochloride to neutralize the potassium hydrosulfide, cystine was not recovered after heating for 19 hours at  $100^\circ\text{C}$ . or 1 week at  $80^\circ\text{C}$ . Recovery was 25 per cent after heating 10 hours at  $105^\circ\text{C}$ ., 67 per cent after heating 10 hours at  $100^\circ\text{C}$ ., and 85 per cent after heating 5 hours at  $100^\circ\text{C}$ .

A tube containing 800 mgm. cysteine hydrochloride and 4 drops of concentrated HCl was suspended in 4 cc. water, containing 17 mgm. of cadmium sulfide, containing radioactive sulfur. The bomb was sealed and heated with agitation until the cadmium sulfide had dissolved. It was then heated for 5 hours at  $100^\circ\text{C}$ . An 85 per cent recovery of cystine was obtained. Radioactivity measurements indicated incorporation of 0.6 per cent of the radioactive sulfur used. After recrystallization 0.13 per cent incorporation was found.

In another experiment, using 17 mgm. of cadmium sulfide, 1600 mgm. of cysteine hydrochloride, 4 drops of concentrated HCl, and heated for 10 hours at 100° C., 67 per cent recovery of cystine was obtained. Activity measurements indicated incorporation of 2.4 per cent of radioactive sulfur. After recrystallization, 0.26 per cent incorporation was found.

#### SUMMARY

Utilizing synthetic methods developed by du Vigneaud and coworkers and by Tarver and Schmidt, three amino acids containing radioactive sulfur were each prepared from 0.06 mole of BaSO<sub>4</sub>. Cystine was obtained in 17 per cent yield, homocystine in 25 per cent yield. The yield of methionine from homocysteine reported by Tarver and Schmidt was not duplicated.

An improved method for preparing benzyl mercaptan from radioactive sulfur is described.

Radioactive sulfur was generously supplied by Dr. Martin D. Kamen, Radiation Laboratory, Berkeley, California.

The work described in this paper was done under a contract, recommended by the Committee on Medical

Research, between the Office of Scientific Research and Development and Harvard University.

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# TRAUMATIC SHOCK. III. A MODIFIED ELECTROSCOPE ESPECIALLY SUITED FOR MEASURING SUBSTANCES WITH LOW ENERGY RADIATION

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Radioactivity measurements have been made with electroscopes and Geiger-Müller discharge counters. In either case, absorption of a percentage of the radiation by the material in the various windows used lowers the sensitivity of the instrument. This is a serious disadvantage in measuring substances with low energy radiation, such as radioactive sulfur (0.107 Mev.). Recently developed Geiger-Müller counters (1, 2) possess an extremely thin mica window, the use of which is made possible by filling the chamber with helium at atmospheric pressure (pressure ionization chamber). However, the electroscope is less expensive and a much simpler type of measuring device for the biological worker who is not sufficiently well-versed in radio-engineering to keep a Geiger-Müller counter in continuous working order.

Henriques and Halford (3) have increased the sensitivity of the Lauritsen electroscope<sup>1</sup> by introducing the specimen to be measured into the fiber chamber, utilizing a sliding bar device, with air lock, in the side of the chamber. In addition, a drying agent (anhydrous magnesium perchlorate) is introduced into the chamber. In this way, precipitates, collected on filter paper (over an area of 1.54 sq. cm.) and placed in a brass cup in the sliding bar, are introduced in a fixed position within the chamber. In the case of sulfur, particularly, care must be taken to measure the radiation from a minimum of material in order to minimize the effects of self-absorption.

We have further modified the electroscope by so placing the introduction slot that the filter paper occupies a position directly beneath the quartz fiber and exactly 1 cm. below it. This increases the sensitivity of the electroscope about 20 per

cent. The construction of the fiber chamber is represented diagrammatically in Figure 1.

In general, counters can be constructed without sensitivity to temperature change, with greater linearity and more constant background than is possible in electroscopes, but the range of activity that can be covered is about the same for both. The error of the electroscope, due to lack of linearity, can be corrected by making standards of known dilutions sufficient to cover the range of radioactivity being measured. The error of the electroscope due to fluctuation in background<sup>2</sup> can be largely avoided by utilizing a working range of at least 3 to 10 times its background. In the tables below, therefore, whenever possible, this range is taken as the zero point in calculating deviations from linearity. Table I illustrates the deviation from linearity of the electroscope and the counter as shown by three sets of standards made up from radioactive sulfur precipitated as barium and benzidine sulfate. The error due to lack of linearity can be partly eliminated by selecting standards with activity close to that of the specimens being analyzed.

In a comparison of this electroscope with a

<sup>2</sup> The daily variation in background amounted to 0.00002 divisions per second. Professor Evans, in a personal communication, has pointed out that ionization devices (electroscopes) have an inherently greater fluctuation in background than Geiger-Müller counters. In the counter, the entering particle or ray produces one count, no matter what its energy content may be. But in the electroscope, the degree of ionization produced by the particle depends on its energy content. The energy content of  $\beta$ -rays from any given artificially produced radioactive substance, varies over a wide range. Since there is an even greater variation in the energy content of background particles due to  $\alpha$ -rays, than in the number per second due chiefly to cosmic rays, greater fluctuation in electroscope background may result. For a mathematical treatment of this subject see Evans (4) and Evans and Neher (5).

<sup>1</sup> Purchased from Fred C. Hanson Company, Pasadena, California.



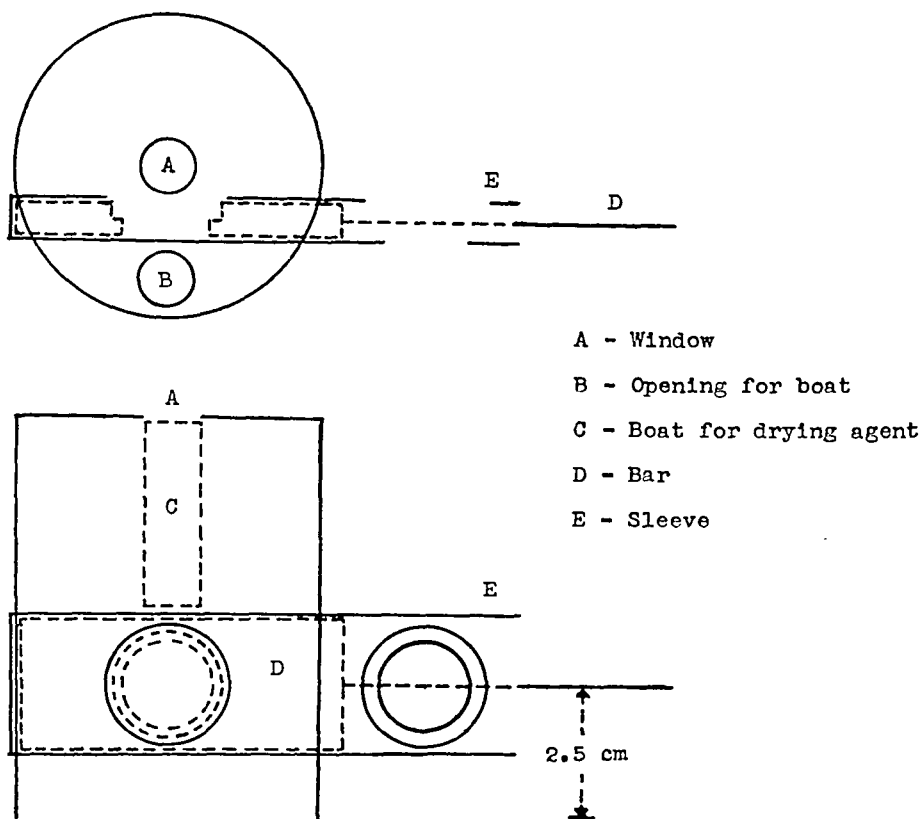
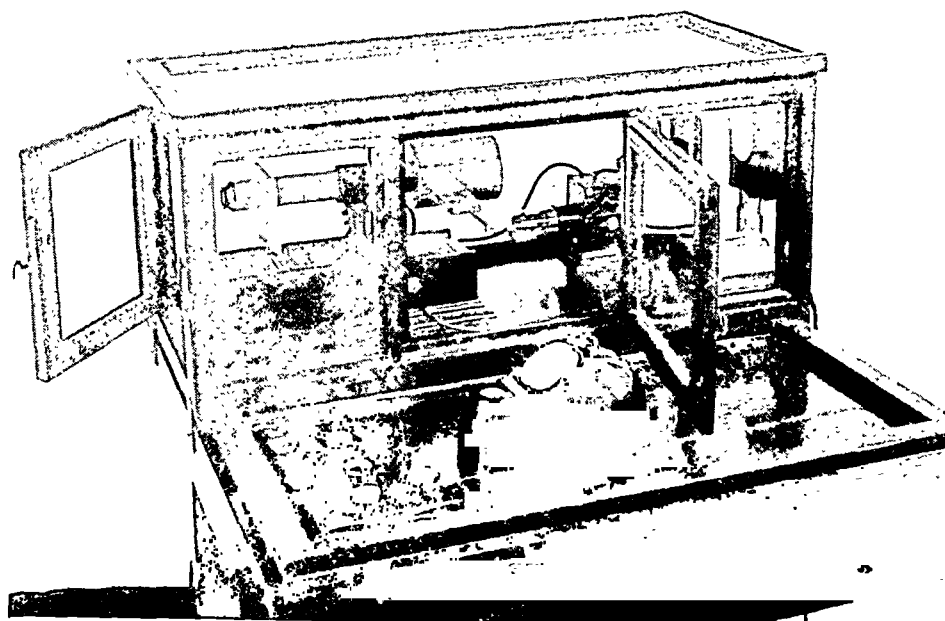


FIG. 1. PHOTOGRAPH AND DIAGRAMATIC REPRESENTATION OF CASE FOR LAURITSEN ELECTROSCOPE

Geiger-Müller counter developed for measuring radioactive sulfur, it was found that the activity of the weakest specimen of sulfur measurable with accuracy in the electroscope was of the same order of activity as the weakest specimen measurable with accuracy in the counter. Measurements of a specimen having 0.1 of this activity were off in both instruments (Table I).

Although primarily developed for measuring the soft radiation of radioactive sulfur, it was found that the electroscope also measures the more penetrating radiation of other elements, accurately and conveniently (Table II).

That most of the error was inherent in the method of oxidation and handling of the silver salt precipitate was shown by comparison of meas-

TABLE I

*Comparison of sensitivity and linearity of electroscope with Geiger counter in measuring radioactive sulfur*

Dilution of radioactive standard	Weight of precipitate*	Electroscope			Geiger counter		
		Radioactivity†	Ratio of radioactivity to background	Deviation from linearity	Radioactivity‡	Ratio of radioactivity to background	Deviation from linearity
	mgm.	divisions per second		per cent	counts per minute		per cent
1	7.6	0.00036	4	0	250	12	0
10	7.4	0.000038	0.5	+5	32	1.6	+28
100	7.0	0.0000021	0.09	-46	10	0.5	+300
1	8.8	0.00047	7	0	260	13	0
10	9.0	0.000064	0.9	+36	28	1.4	+7
100	8.9	0.000013	0.18	+175	5	0.2	+90
1	10.5	0.033	250	-13			
25	9.7	0.0015	10	-1.3			
100	9.5	0.00038	3	0	165	16	0
1000	9.3	0.000028	0.2	-26	18	2	+9

\* The radioactive standard was diluted with inert sulfate before precipitation.

† BaSO<sub>4</sub> precipitate.

‡ Benzidine sulfate precipitate.

§ One division per second represents 20 small scale divisions of the electroscope, at least 4 small scale divisions were clocked in each determination.

¶ Data provided by Dr. Frederick Henriques, Harvard University, and Dr. Robley Evans, Massachusetts Institute of Technology.

measurements of plasma treated by both the oxidation technique and evaporation to dryness (Table III). The greater deviation from linearity shown with oxidized specimens in Table III as compared to Table II may be due to an excessive weight of silver precipitate.

TABLE II

*Illustrating the linearity shown in 2 sets of standards prepared from radioactive bromine precipitated as silver bromide*

Dilution of radioactive standard	Weight of silver bromide precipitate*	Radioactivity	Ratio of radioactivity to background	Deviation from linearity
	mgm.	divisions per second†		per cent
1	16.7	0.0074	60	+27
10	14.8	0.00058	5	0
100	14.9	0.000064	0.5	+10
1	13.8	0.0041	35	-14
10	16.5	0.00048	4	0
100	15.5	0.000080	0.7	-17

\* The radioactive standard was diluted with inert bromide before precipitation.

† One division represents 20 small divisions on the scale of the electroscope. At least 4 small divisions were clocked in each determination.

It was found when radioactive bromine possessing a high order of radioactivity (approximately 0.3 divisions per second) was measured in the electroscope, the background of the instrument was affected unfavorably for a period of weeks to months. In one case, the background changed from 0.00006 divisions per second to 0.0005 divisions per second. That the change was not due to foci of secondary radiation in the electroscope chamber was shown by transferring the chamber to another electroscope. The change was probably due to ionization of the insulating material supporting the fiber, allowing leakage from the charge on the fiber. On 2 occasions, a spontaneous return to normal background occurred over a period of weeks. The phenomenon exhibited a threshold; for repeated measurements of potent specimens of a lesser order of activity did not alter the background of the instrument. It is therefore wise to have on hand a second electroscope, if specimens of a high order of activity are to be dealt with. In the routine use of the instrument, the fiber was charged at least one hour before use to saturate the insulator material.

TABLE III  
*Comparison of measurements of plasma treated by both oxidation and evaporation*

Dilution of radioactive standard	Oxidation method				Evaporation method	
	Weight of silver bromide precipitate	Radioactivity	Ratio of radioactivity to background	Deviation from linearity	Radioactivity	Deviation from linearity
	<i>mgm.</i>	<i>divisions per second</i>		<i>per cent</i>	<i>divisions per second</i>	<i>per cent</i>
1	25	0.0356	500	-51	0.0769	-2
2	25	0.0227	320	-34	0.0416	+6
4	24	0.0146	200	-11	0.0192	-2
8	23	0.0092	130	+15	0.0099	+1
16	22	0.00403	57	0	0.0049	0

#### SUMMARY

A modification of the Lauritsen electroscope, which increases its sensitivity, is described. This makes it possible to measure the soft radiation from radioactive sulfur with an accuracy comparable to that of Geiger-Müller counters constructed for the same purpose. Other radioactive elements may also be measured with this instrument.

It is expected that this simple instrument will be particularly useful to biological investigators unable to procure and maintain in continuous running order the more complicated Geiger-Müller counter.

We are indebted to Dr. Frederick Henriques of Harvard University, and Professor Robley D. Evans of Massachusetts Institute of Technology, for the comparisons with their Geiger-Müller counters. Dr. Henriques

gave valuable advice and assistance in the construction of the electroscope chamber.

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# TRAUMATIC SHOCK: IV. A STUDY OF THE PROBLEM OF THE "LOST PLASMA" IN HEMORRHAGIC SHOCK BY THE USE OF RADIOACTIVE PLASMA PROTEIN

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A prevailing opinion is that an increased permeability of the capillaries exists in shock and that a consequent loss of plasma into tissues ensues, so that the effective circulating blood volume falls to a level incompatible with life. This loss of plasma is presumed to occur generally throughout the body as well as in areas of local injury, because the replacement of all or more than can be accounted for as lost at the site of injury is unable to sustain the organism. The evidence in favor of this theory is derived from the following: (1) There is peripheral stagnation of circulating blood, as shown by hemoconcentration, cyanosis, lowered venous oxygen, decreased venous return to the heart, decreased pH of venous blood, and prolonged circulating time; (2) the tissues are wet or hemorrhagic; and (3) the circulating plasma volume, as determined principally by dye methods, is reduced sharply.

The difficulty in accepting this evidence as conclusive is that fatal shock may occur in the absence of some of the above-mentioned features. For example, hemorrhagic or edematous tissues and hemoconcentration are not usually found in fatal shock resulting from simple hemorrhage. When they do occur, they may be explained as phenomena of tissue deterioration in a dying organism, whatever the cause, more especially if an abundance of intravenous fluids has been supplied. The decline in effective circulating volume and in the volume of venous return to the heart are constant findings, but they are readily accounted for by the loss of blood in the case of shock from hemorrhage, or the escape of plasma into areas of injury in such types of shock as trauma, burns, infections, and intestinal strangulation. The fact, however, that adequate replacement of a known or calculated plasma loss will reverse the deteriorating trend in shock if given sufficiently early, but not if given late, has led

to the inference that the plasma escapes everywhere from the circulation in the late or irreversible phase because of capillary "leakage." It is, moreover, clear that a fatal loss of plasma (40 per cent), if generally and evenly distributed throughout the body, may not be detectable by ordinary methods of measurement. Hence, the elusive nature of the "lost plasma." Obviously, it is essential to establish or deny the claims for the "lost plasma" hypothesis if we are to be properly oriented, not only as to the mechanisms involved, but as to the direction by which improvement in therapeutic techniques may be achieved.

Since the incorporation of a radioactive element into the plasma protein molecule provides an unmistakable label for the identification of plasma proteins, which presumably would be lost with the non-protein fraction, the use of such radioprotein was considered a more promising technique for the solution of the "lost plasma" problem than other available methods.

Such labelled proteins are valid tools so long as they are not denatured in preparation or do not behave like a foreign protein when used in the experimental animal. Accordingly, sulfur, a natural constituent of plasma protein, was first selected as the element to be made radioactive for incorporation into the protein. The special problems involved in this synthesis have been fully discussed in preceding papers of this series (1, 2). The prepared radioactive sulfur-containing plasma protein has been sufficient for study of its disappearance rate upon injection intravenously into the normal and shocked dog, but extensive observations could only be made with extraordinary difficulty because, (1) radioactive sulfur can be obtained only occasionally and in small amounts, (2) the synthesis of radiosulfur-containing L-cystine can be achieved at best with only an 8 per cent yield on the basis of sulfur,

and (3) the yield of plasma protein is only 15 per cent on the basis of radioactive 1-cystine fed. For this reason, radiobromoprotein, which can be prepared relatively easily (as will be described below) was utilized for the bulk of the observations. This was possible because it was found that radiobromoprotein, containing less than 0.1 per cent bromine, possessed nearly as slow a disappearance rate from the blood stream of normal dogs as radiosulphprotein.

This communication is concerned with a study of the movement of plasma and plasma protein from the blood stream into the tissues in the normal dog and in the dog shocked by hemorrhage. The data at hand contradict the theory that a progressively increasing loss of plasma from the circulation occurs in shock due to hemorrhage.

#### METHOD

##### *Preparation of plasma protein containing radioactive sulfur*

The preparation of radioactive plasma protein from radioactive sulfur-containing cystine by hypoproteinemic dogs, was described in a preceding paper (2). Radioactive plasma was removed from these dogs and dialysed for 36 hours in running tap water. In order to prevent the extensive precipitation of globulins which are redissolved with difficulty, 1.0 cc. of 25 per cent sodium carbonate per 10 cc. plasma was added before dialysis was begun. Dialysis was continued in physiological saline as soon as precipitation occurred. When desired, plasma was concentrated by evaporation in a cellophane bag with electric fan ventilation. Following dialysis, radioactivity of the non-protein fraction of plasma represented 0.14 per cent of the total radioactivity. The preparation of tissue and plasma containing radioactive sulfur for radioactivity measurement is described in paper I of this series (2).

##### *Preparation of radioactive bromoprotein*

Radioactive bromine was obtained from ethyl bromide after neutron bombardment, by two extractions with 400 cc. each of 2 to 3 per cent sodium carbonate. Two per cent sodium sulfate was added to prevent emulsification. The aqueous extract was then extracted with ether to remove ethyl bromide.<sup>1</sup> A few pellets of sodium hydroxide were added to the aqueous extract (some 800 cc.), which was then evaporated to 100 cc., cooled and

neutralized with cold 50 per cent sulfuric acid. The solution was then transferred to an all-glass distilling apparatus with water cooled condenser, ice-cooled water trap, and final trap containing a small amount of 25 per cent sodium carbonate solution. A mixture of powdered manganese dioxide in concentrated sulfuric acid was added in excess, and the mixture was heated to boiling. An estimate of the quantity of bromine obtained was made from the volume of bromine and bromine water.

The cold bromine and bromine water were added at once with swirling to 10 to 15 cc. of a cold 25 per cent sodium carbonate solution. The resulting yellow solution of sodium hypobromite was added immediately to 30 to 60 cc. of cold plasma. After thorough mixing, it was allowed to stand at room temperature for 45 minutes. An aliquot of this solution was taken for determination of the radioactivity of the total bromine used in bromination of the plasma. The remaining solution was placed in cellophane tubing (1½ inches in diameter) which was ligated with 3 ties, close to the top of the column of solution, to prevent too large uptake of water. It was then dialysed in running tap water for 36 hours. The bag was slowly rotated during this time. Only a slight amount of precipitate formed during the dialysis. This was centrifuged and the supernatant fluid used in the experiments to be described.

An aliquot was taken for determination of the amount of radioactive bromine attached to protein. The activity of the non-protein fraction was determined with the filtrate obtained after precipitation with an equal volume of 20 per cent trichloroacetic acid. Values varying from 0.2 to 0.8 per cent of the total activity of the plasma were found in this non-protein fraction. With these data, it was possible to calculate the percentage of the bromine used in the bromination which was attached to the plasma protein. This varied from 20 to 30 per cent.

From the approximate weight of bromine used in bromination, the percentage utilization, and the plasma protein concentration, the percentage bromine in the bromoprotein was calculated. This varied from 0.05 to 5.0 per cent. From the estimated minimal molecular weights (3) of serum albumin (45,000) and serum globulin (81,000), it is evident that if one atom of bromine (82) is incorporated in each protein molecule, the resulting percentage of bromine in bromoalbumin would be 0.18 per cent and in bromoglobulin 0.10 per cent. Therefore, in order to tag a maximum number of protein molecules with a minimum number of radioactive bromine atoms, the optimal percentage of bromine in the bromoprotein desired should be 0.1 to 0.2 per cent. Bromoprotein containing 5 per cent bromine would contain 35 bromine atoms on each protein molecule.

When the bromination was conducted in stronger alkali, a more denatured soluble protein was obtained. Equally efficient bromination of protein was obtained with sodium hydroxide as with sodium carbonate, but evidence of denaturation of the protein appeared from the fact that protein disappeared from the circulating plasma in normal animals at an abnormal rate. Ammonium hydroxide was

<sup>1</sup> Unless this procedure is followed, or if stronger alkali is used, excessive hydrolysis of ethyl bromide—1.0 to 1.5 grams bromine from 35 kgm. ethyl bromide—results. Wet ethyl bromide was stored in the cold. By the procedure outlined, as little as 20 to 40 mgm. bromine are obtained from 35 kgm. of ethyl bromide.

unsatisfactory because it rapidly decomposed the sodium hypobromite.

*Stability of the bromine linkage in radioactive bromoprotein*

In order to determine the ease with which bromine could be removed from its points of attachment in the protein molecule, a specimen of radioactive dialysed plasma was hydrolysed with trypsin and sulfuric acid.

*Trypsin hydrolysis.* To 2.0 cc. of plasma were added 15 mgm. of crystalline trypsin and the mixture was incubated at 37° C. for 3 hours. A very slight precipitate was obtained when an equal volume of 20 per cent trichloroacetic acid was added.

*Sulfuric acid hydrolysis.* Plasma (2.0 cc.) and 6.0 cc. of 25 per cent sulfuric acid were heated in a sealed tube at 70° C. for 7 hours. A slight precipitate was obtained when an equal volume of 20 per cent trichloroacetic acid was added.

To each filtrate obtained as described above, and to the non-protein filtrate prepared in the same way from 2.0 cc. of plasma, potassium bromide equivalent to 7.0 mgm. of silver bromide was added, followed by an excess of silver nitrate. The precipitates were collected and the radioactivity of each was determined.

The plasma was found to contain 0.85 per cent of its radioactivity as ionic bromide. After trypsin hydrolysis, this increased to 2.6 per cent and after sulfuric acid hydrolysis, it increased to 3.0 per cent. Analysis of the urine of a dog which was given 30 cc. of this specimen of dialysed radioactive plasma intravenously, showed an excretion (as ionic bromide) of 1.3 per cent of the radioactivity which was injected, in a 12-hour period.

In another experiment, 100 cc. of radioactive dialysed plasma, containing 0.5 per cent of its radioactivity as ionic bromide, was given intravenously to a dog. Analysis of pooled plasma specimens, collected over a period of 12 hours, showed a content of 3.7 per cent of the radioactivity in the ionic bromide fraction.

In still another experiment, pooled plasma from 3 dogs (normal and shocked), one half hour after injection of radioactive bromoprotein, showed 0.07 per cent of the radioactivity in the non-protein fraction and 5 hours later the non-protein fraction contained 1.1 per cent of the plasma radioactivity.

This experimental evidence indicated that some 3.0 per cent of the radioactive bromine incorporated in the plasma protein could be liberated by *in vitro* or *in vivo* hydrolysis, but that some 97 per cent was firmly found in one or more amino acids, presumably tyrosine and possibly tryptophane (4). It is expected that bromine in such a linkage would be liberated by nothing less than destruction or degradation of the aromatic ring holding the bromine atom.

*Preparation of plasma and tissues containing radioactive bromine for radioactivity analysis*

Because bromine emits a  $\beta$ -ray of sufficient penetrability (0.7 Mev.), it is possible to prepare plasma, lymph, or urine for radioactivity measurements simply by evapora-

tion to dryness. For accurate comparison of specimens, the dry weight of the specimens should be approximately the same.

For analysis of tissue for content of radioactive bromine, it was found desirable to oxidize the specimen in the presence of silver nitrate and measure the radioactivity of the resulting silver halides. The weight of silver halides was kept constant as far as possible (see below). For accurate comparison of plasma and tissue radioactivity, the silver salt method was used for both.

Each specimen for analysis (1 gram) was oxidized in a 100 cc. Kjeldahl flask with nitric acid and superoxol in the presence of an excess of silver nitrate (equivalent to 30 mgm. silver bromide). A standard weight of potassium bromide (equivalent to 7.0 mgm of silver bromide) was added to each flask. When standard dilutions of plasma were oxidized, chloride content was made up by adding 1.0 cc. of normal plasma or 0.5 cc. of 0.9 per cent saline solution.

The oxidation required 1.5 to 2.0 hours. Care was taken to avoid ignition when approaching dryness. Nitric acid and superoxol were added in small quantity alternately until an ash was formed. Heating was continued until nitric fumes disappeared. Fumes were removed by water pump aspiration through a tube which fitted loosely over the top of the Kjeldahl flask. The resultant yellowish-white ash was dissolved in 15 to 20 cc. of concentrated ammonium hydroxide. This was facilitated by leaving the ash in the ammonia overnight. A small amount of white ash (usually noted with tissue such as liver and kidney) failed to dissolve. This was ignored, since it was found to be acid soluble (probably phosphate). The solution was then transferred to a 50 cc. beaker, with the aid of additional ammonia. The solution was evaporated smoothly on a hot plate until a precipitate began to form. Two to 3 cc. of 20 per cent nitric acid were then added and the evaporation continued to 3 to 4 cc. An additional 2 to 3 cc. of 20 per cent nitric acid was added and the mixture was allowed to remain in the dark and cold for one half hour. The precipitate was then collected over an area of 1.54 sq. cm. in a filtration apparatus already described (2). The precipitate was washed with water, acetone, and finally, an acetone solution of vinylite, and sucked dry. Care was taken to secure a layer of uniform thickness. It was found that the vinylite film agglutinated the silver salt particles into a flexible, easily handled layer. Precipitates weighing 14 to 16 mgm. were regularly obtained.

To test the reliability of this method of oxidation and precipitation, standard dilutions of radioactive bromoprotein were made with normal plasma. Comparative measurements of radioactivity were made with both evaporated and oxidized specimens (Table III of foregoing paper). Greater deviation from linearity was noted in oxidized specimens (consistent loss).

*Measurement of radioactivity*

Radioactivity measurements were made with a modified Lauritsen electroscope described in the preceding paper

(5). Plasma (1.0 cc.) was evaporated in an electric oven at 85° to 90° C. in shallow brass cups of uniform size, which were introduced in a fixed position into the electroscope chamber by means of a sliding bar device. Precipitates of silver bromide on filter paper were also introduced in brass cups in the same manner. Parallel "evaporated" and "oxidized" specimens of standards of radiobromoprotein solutions used for injection were read, so as to permit tissue calculations.

All standards and specimens were measured on the same day, the time of measurement of each was recorded, and the decay for the total period of electroscopic analysis determined by repeating the measurement of the first specimen. Radioactivity in division per second for each specimen was corrected to zero time. When such measurements were made, as was usually the case, 5 to 6 days after the preparation of the radioactive bromine, short half-life bromine had deteriorated to such an extent that the rate of decay corresponded fairly well with the rate of decay of 34-hour half-period bromine ( $\text{Br}^{82}$ ).

*Rate of disappearance of radioactive plasma protein from plasma of normal dogs*

In the course of the experiments to be reported, radioactive plasma was given to normal anesthetized and unanesthetized dogs, intravenously. The rate of disappear-

ance of radioactive protein from the circulating plasma was determined in each case. The percentage of the radioactive protein injected, which was found circulating in the plasma, was calculated from the unit radioactivity of plasma, the plasma volume, and the total radioactivity of the protein injected.

In the case of the plasma protein containing radioactive sulfur, 90 per cent of the radioactive protein was circulating 5 hours after injection, 70 per cent, 15 hours after injection, and 45 per cent, 48 hours after injection in two experiments.

The results with plasma protein containing radioactive bromine were more variable, depending upon the degree of denaturation of the protein. Denaturation was increased by increasing the bromine content of protein and by bromination in strong alkali. Figure 1 is a composite of these results with radioactive plasma protein in normal anesthetized dogs. The slowest rate of disappearance of radioactive bromoprotein comparable to the rate of disappearance of radioactive sulfur-containing protein was obtained with bromoprotein having less than 1 per cent bromine and prepared with sodium carbonate. Because of the variation in the rate of disappearance from the circulation of bromoprotein with different bromine percentages, and because of the impossibility of preparing radioactive bromoprotein of constant bromine composition, it

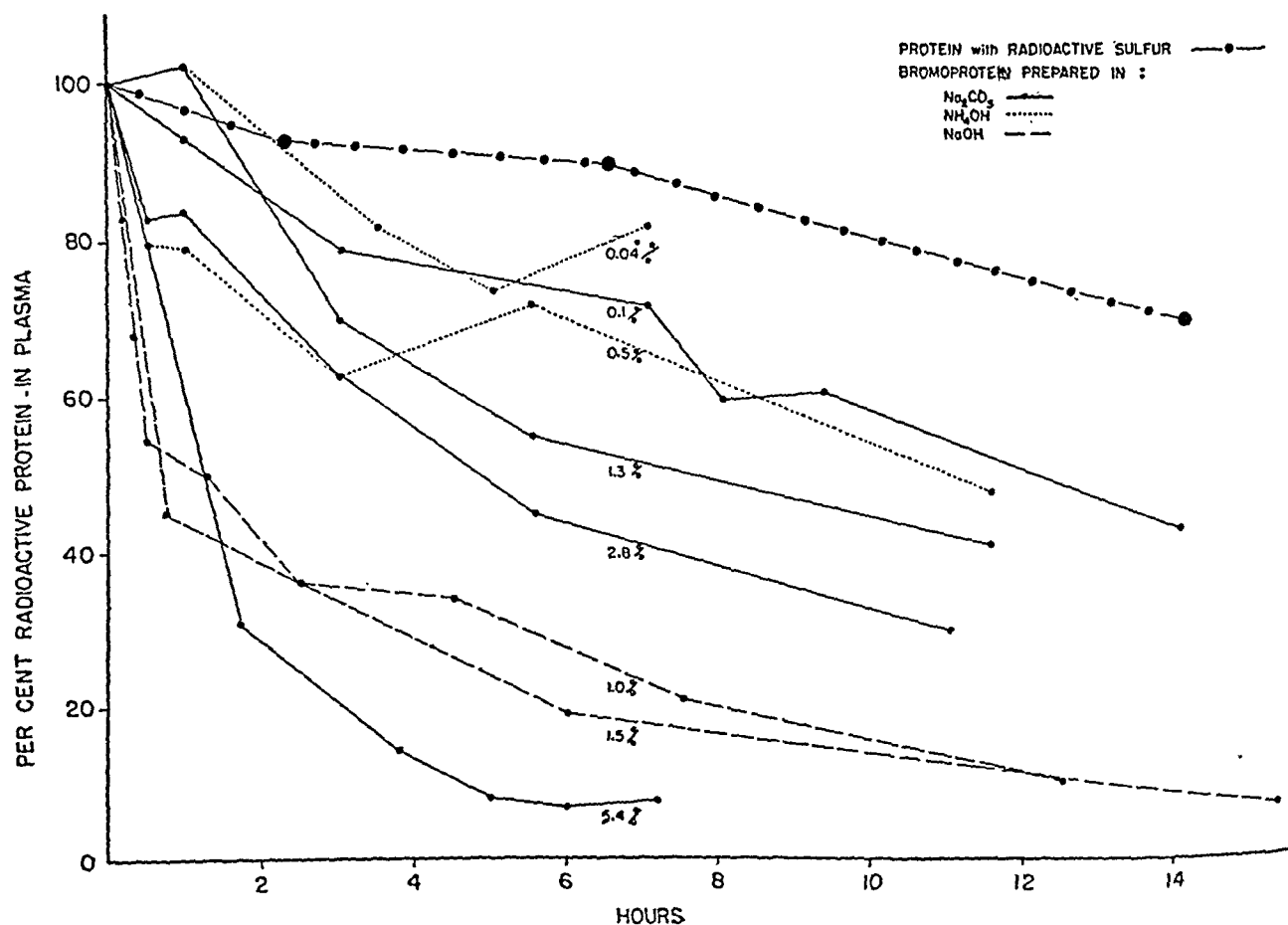


FIG. 1. RATE OF DISAPPEARANCE OF RADIOACTIVE BROMOPROTEIN (CONTAINING VARIOUS PERCENTAGES OF BROMINE AND PREPARED IN SEVERAL WAYS) FROM THE CIRCULATING PLASMA OF NORMAL ANESTHETIZED DOGS, COMPARED TO RADIOACTIVE PLASMA PROTEIN CONTAINING RADIOACTIVE SULFUR

was necessary to use a normal dog as a control in every shock experiment in which plasma containing radioactive bromoprotein was used for the study of the movement of plasma. No difference was noted between anesthetized and non-anesthetized dogs in the rate of disappearance of this bromoprotein from the plasma.

*A comparison of shocked and normal dogs in the rate of disappearance of radioactive plasma protein from the circulation and the distribution of radioactive protein in the tissues*

**Procedure.** Dogs were anesthetized (3 exceptions) with morphine sulfate (3 mgm. per kgm.) and sodium barbital, according to Wiggers' technique (6). In three cases, morphine sulfate (2 mgm. per kgm.) only was given and local anesthesia was used. Plasma volumes were determined by the method of Gibson and Evelyn (7). Plasma total protein was determined by the method of Kagan (8). In most cases, bleeding was accomplished as follows: A massive withdrawal of blood corresponding to 1 to 2 per cent of the body weight was made, followed by successive smaller withdrawals at 20 minute intervals, until a blood pressure of 70 mm. Hg was reached. No further blood (except analytical samples) was removed. Blood pressures were taken by needle puncture of the femoral artery. All injections were made intravenously. All blood samples (heparinized) were taken from the femoral artery.

The amount of blood in each tissue, analysed for radioactive protein, was also determined by extraction of hemoglobin with water. A convenient method of doing this was to let the finely minced tissue stand in water for 48 hours in the icebox. After appropriate dilution, hemoglobin was determined by the benzidine method (9). Blood diluted 10,000 times was used as standard. On the basis of the hemoglobin content of each tissue and the arterial hematocrit at the conclusion of the experiment, the approximate intravascular plasma content of the tissue was calculated. Although it is recognized that the arterial differs from the capillary hematocrit, the error introduced, so far as intravascular plasma content of tissue is concerned, is small. Experiments are in progress, however, to determine the discrepancy between arterial and capillary hematocrit in normal and shocked dogs. On the basis of the unit radioactivity of the circulating plasma at the end of the experiment, the radioactivity of the intravascular plasma in 1.0 gram of tissue was calculated. This was used to calculate the extravascular radioactive protein in 1.0 gram of tissue.

When the animals had been exsanguinated (the usual procedure in order to reduce blood content of tissues), the correction for blood content of tissue in skin, bowel, brain, and omentum was too small to be significant. The determination of the hemoglobin content of skeletal and heart muscle gave high values because of the presence of myoglobin. There are very few red cells in the physiological saline used for extraction of blood from carefully minced muscle, as compared to the number found in other tissues, such as lung, similarly treated.

The correction for blood in any case would only be necessary in muscle showing plasma loss. Since this occurred only twice in 12 instances (see Table II), no correction for blood in muscle need be applied. Corrections for blood in liver, kidney, and lung were necessary because of considerable variations in blood content.

Radioactive plasma was given intravenously to a normal and a shocked dog simultaneously, some time before or immediately after the shocked dog had been bled. The dose was the same in proportion to body weight in both dogs except in Experiments 8 A and 8 B. The volume was usually too small to affect the total volume significantly. If the volume of plasma injected was over 25 cc., blood was withdrawn to balance that given. The disappearance curve of the radioactive protein was determined by sampling at intervals until the death of the shocked dog. In several instances, the latter received intravenous saline or plasma late in shock. In some experiments, only disappearance curves were studied and in others, tissues were taken for radioactivity measurements immediately after death following exsanguination. The disappearance curves (Figures 2 to 8) are shown in percentage of radioactive protein given, circulating in plasma as a function of time, as well as in terms of radioactivity per cc. of plasma as a function of time. The percentage of the radioactive protein injected which was circulating in plasma was determined from the measured or estimated plasma volume and the unit activity of plasma from the formula:

$$\text{Percentage radioactive protein in plasma} = \frac{\text{Plasma volume} \times \text{radioactivity per cc. plasma}}{\text{Radioactivity injected}} \times 100$$

The radioactive protein removed with the withdrawn blood was corrected for at any given time by the formula:

$$\text{Percentage radioactive protein in plasma} = \frac{\text{Plasma volume} \times \text{radioactivity per cc. plasma}}{\text{Radioactivity injected} - \text{radioactivity removed}} \times 100$$

When the radioactive protein was given to both dogs before the shocked one had been bled, the total circulating radioactive protein in the shocked dog after bleeding was of course reduced by the percentage of the total plasma withdrawn, but the concentration remained about the same in both animals, except for a slight decrease in unit activity caused presumably by dilution resulting from mobilization of extravascular fluids. When the radioprotein was given to both dogs after the shocked one had been bled, the total circulating protein given was the same in proportion to the body weight in both dogs. In that case, the smaller circulating volume of the shocked dog resulted in a greater unit activity. When the dose was given in proportion to volume of circulating plasma, the unit activity was the same in both. Since the tissue accumulation of radioactive protein is presumably proportional to the unit activity under ordinary conditions of capillary permeability, a comparison of the radioactivity content of the tissue of the normal and shocked dogs required correction in accordance with



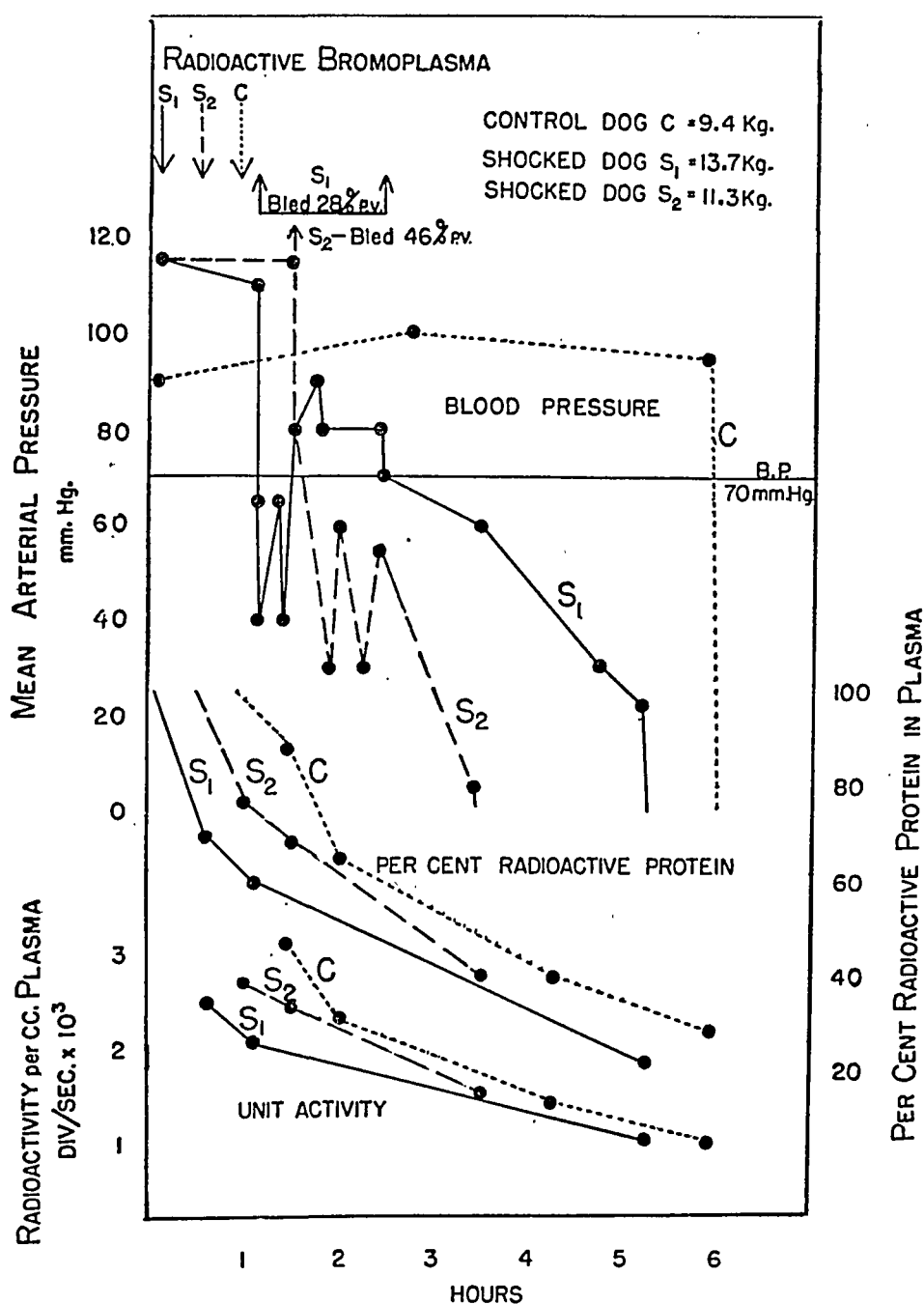


FIG. 2. EXPERIMENT 13

Since the slopes of the radioactivity curves in all three dogs show an approximately parallel course, it follows that the shocked dogs did not lose radioactive protein from the plasma at a greater rate than the control dog.

See Table II for tissue analyses. No evidence of plasma protein loss (within the limits of error) was noted in the shocked dog S<sub>1</sub>, as compared to the control dog.

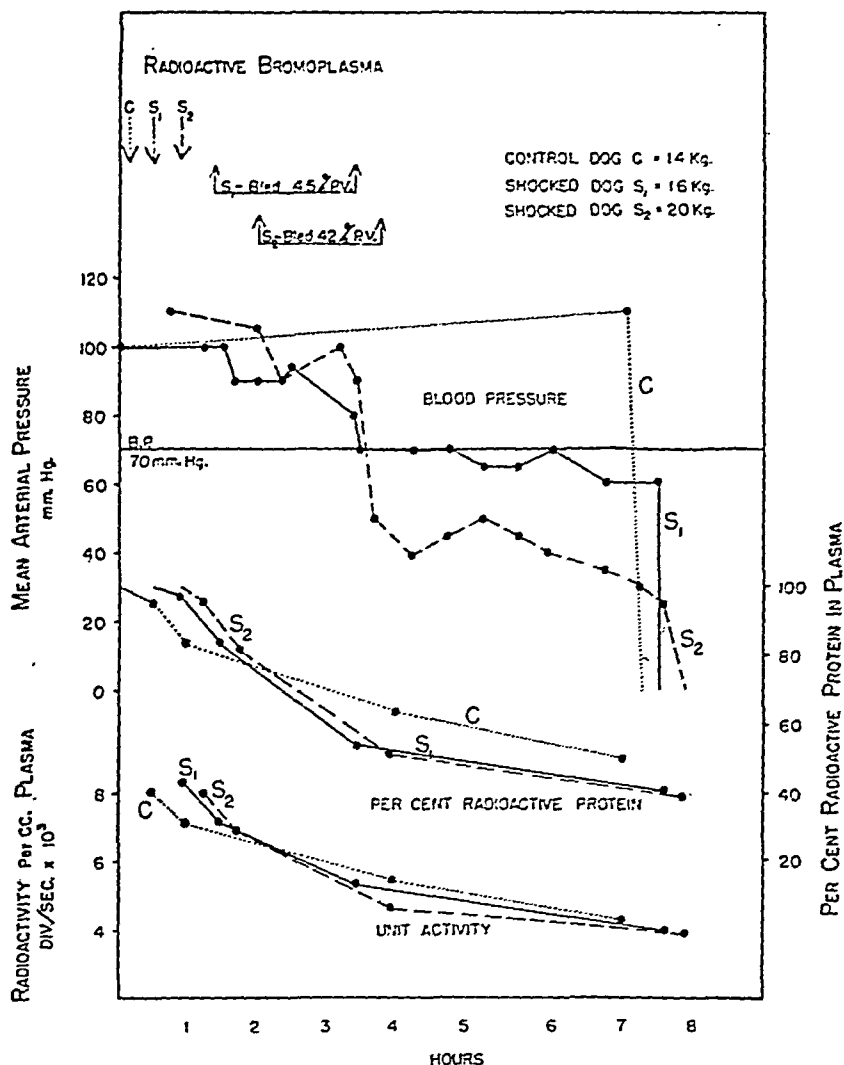


FIG. 3. EXPERIMENT 11

The increased slope of both radioactivity curves for dogs S<sub>1</sub> and S<sub>2</sub>, as compared to that of dog C, immediately after bleeding, is probably due to dilution by mobilized extravascular fluid. Their subsequent parallel course indicates that the shocked dogs did not lose radioactive protein from the plasma at a greater rate than the control dog. There is no difference in the disappearance curves between the dog in deep shock (S<sub>2</sub>, probably irreversible) and the dog in less severe shock (S<sub>1</sub>, probably reversible).

See Table II for tissue analyses. Except for kidney (S<sub>1</sub>) and thoracic skin (S<sub>1</sub> and S<sub>2</sub>), no evidence of plasma protein loss in the shocked dogs as compared to the control dog was noted. The dog in deeper shock (S<sub>2</sub>) showed no significant increase in tissue radioactivity as compared to dog S<sub>1</sub>.

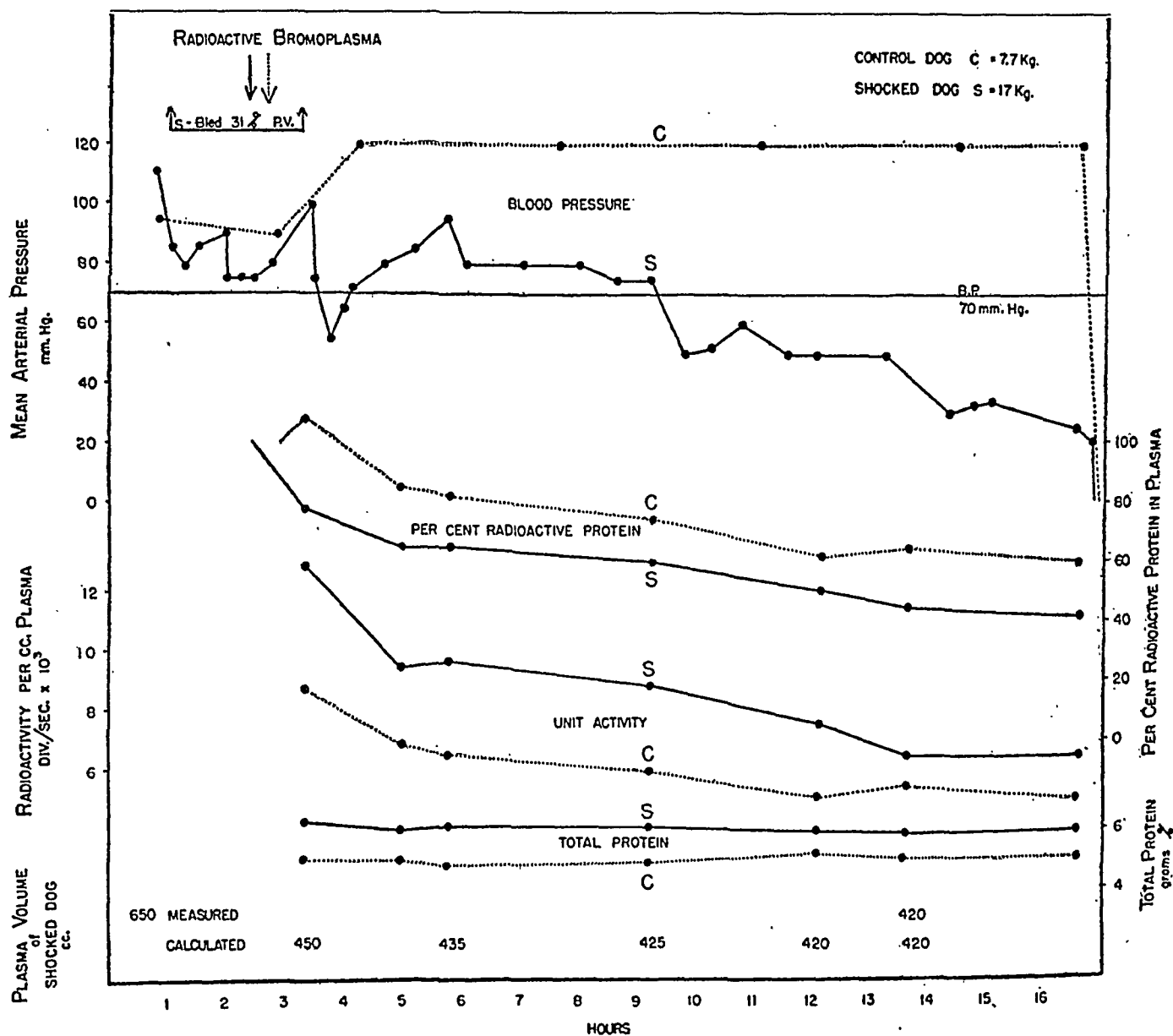


FIG. 4. EXPERIMENT 7

No evidence of significant hemoconcentration or hemodilution is obtained from the data. There is no greater rate of disappearance of radioactive protein from the circulating plasma in the shocked dog as compared to the control dog. No progressive loss of plasma was found with the dye method. The plasma volume 10 hours after hemorrhage is the same as that calculated on the basis of plasma removed in hemorrhage and sampling.

See Table II for tissue analyses. Plasma loss into ileum and liver was found.

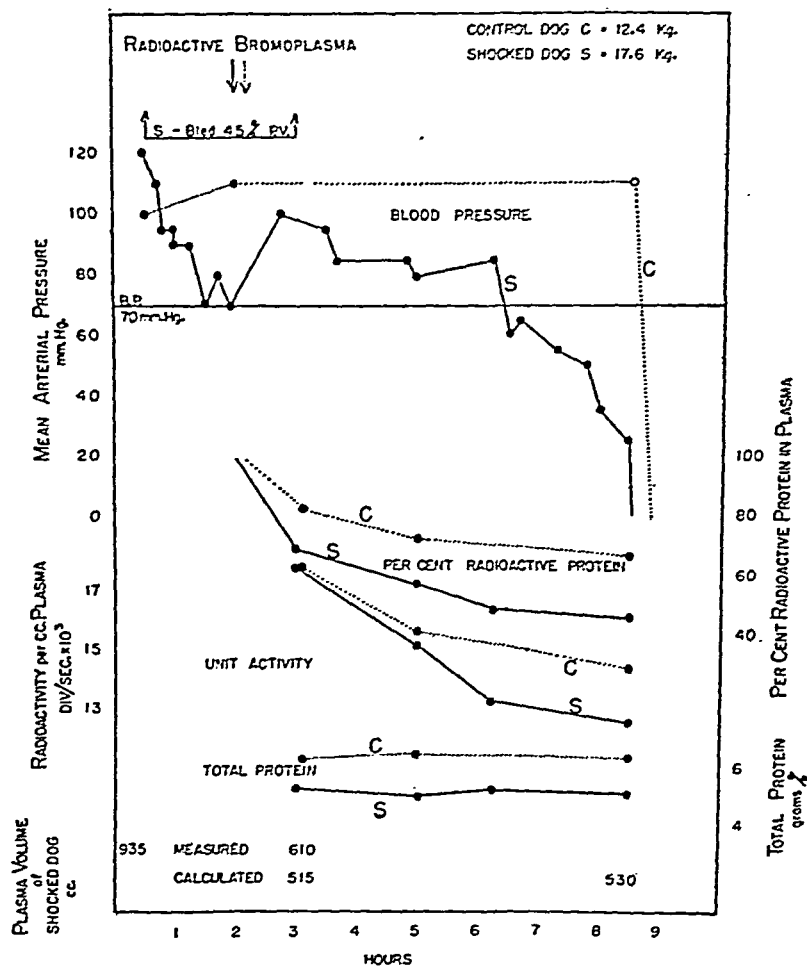


FIG. 5. EXPERIMENT 8

Evidence of slight hemodilution after hemorrhage is obtained from the total protein and radioactivity curves, as well as plasma volume measurements.

See Table II for tissue analyses. No plasma loss was found.

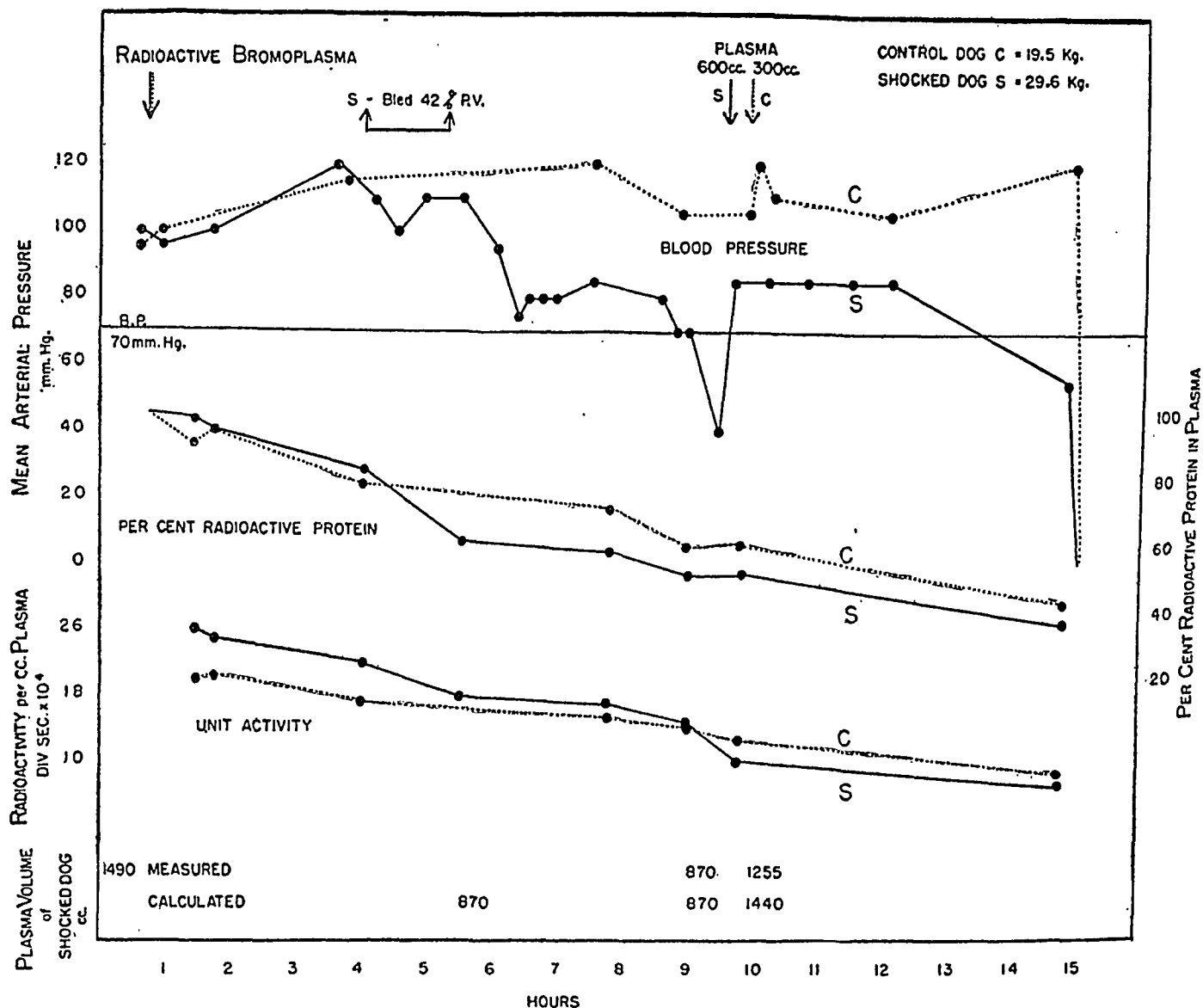


FIG. 6. EXPERIMENT 9

The parallel slopes of the radioactivity curves in shock and after a plasma infusion, indicate no greater loss of radioactive protein in the shocked dog than the control dog. Plasma volume measurements show no loss of plasma, except for that removed by hemorrhage, up to the time of the infusion. A deficiency of 185 cc. of plasma from the circulating plasma was found after the infusion. Since no change in concentration of radioactive protein was noted after the infusion had mixed, whole plasma must have been trapped out of active circulation or lost into tissue.

See Table II for tissue analyses. Loss of plasma into liver, skin, and leg muscle was found.

A similar experiment with 3 shocked dogs and 2 controls (Exp. 10 and 12, Table I) gave the same results. Radioactive protein disappeared from the circulating plasma at the same rate for both shocked and control dogs before and after plasma infusions. No tissue analyses were done in these dogs.

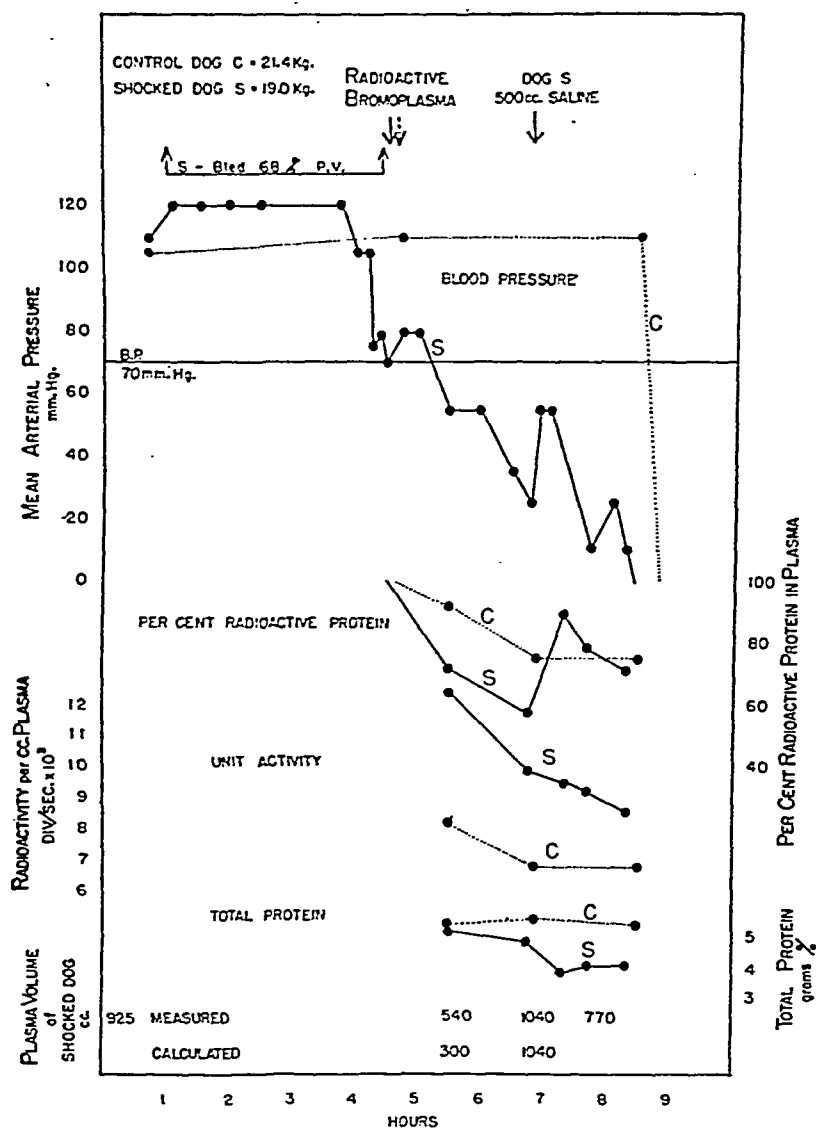


FIG. 7. EXPERIMENT 8A

Evidence of slight hemodilution is obtained from the plasma total protein and radioactivity curves, and plasma volume measurements early in shock. Marked dilution occurred after the infusion.

The percentage of radioactive protein circulating in the plasma increased after the saline infusion to a level higher than was noted immediately after the injection of the radioactive protein. This can only mean that due to poor mixing of the injected plasma during shock, some concentrated plasma was held stagnant in capillaries until it was washed into the circulation by the infusion.

See Table II for tissue analyses. If the plasma lost is assumed to have the activity noted at the end of the experiment, the difference in tissue activity (corrected) between control and shocked dog permits a calculation of the amount lost in liver to be 180 cc., in lung 40 cc., in skin 40 cc., and in leg muscle 27 cc. of plasma (total 287 cc.). Although the error introduced by this assumption gives higher values than probable, the data suggest loss of plasma protein into tissue following a saline infusion in late hemorrhagic shock.

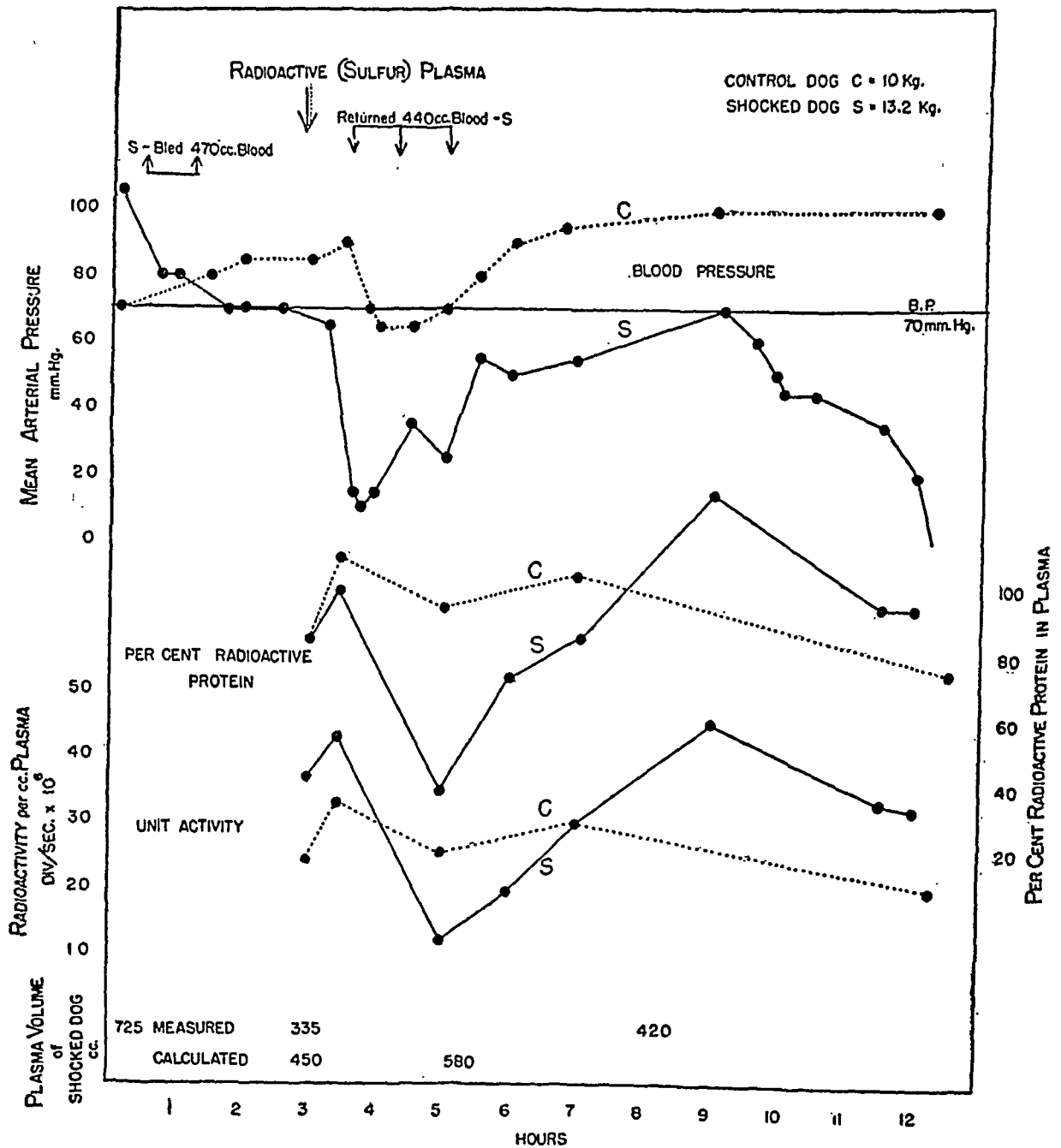


FIG. 8. EXPERIMENT S-1

Radioactive plasma containing radioactive sulfur was used. A plasma volume determination in the shocked dog at the beginning of the experiment, and just before the terminal decline in blood pressure, showed a deficiency of nearly 300 cc. of actively circulating plasma, even though nearly all the blood had been returned.

Inspection of the radioactivity curves shows a rapid loss of radioactive protein during the period of rapid collapse, and mobilization of radioactive protein into the active circulation after transfusion and rise in blood pressure had occurred. This finding is similar to that of experiment 8A, Fig. 7. Evidence of mobilization of radioactivity was not noted in experiments in which the radioactive protein was given before hemorrhage, when adequate mixing with the circulating plasma was allowed (Fig. 6). It is therefore possible that the observed phenomenon is due to inadequate mixing of radioactive plasma, rather than to loss through capillary leakage.

The plasma deficiency observed by the dye method is 37 per cent of the original volume. This is much higher than observed in any other experiment involving transfusion in the late shock phase (Table III). This suggests that the deficiency in part at least may have been due to poor mixing of dye. That inadequate mixing existed in this experiment was evident from the curve of radioactivity following injection of radioactive protein.

Another experiment (Experiment S-3, Table I) was done in which radioactive plasma containing

their weight, the existing differences in unit and total radioactivity of plasma, the volumes of hemorrhage, and of transfusion.

This correction<sup>2</sup> assumes that the plasma volume is variable (hemorrhage or transfusion), and that the capillary bed is about constant in area for any one animal. Equating the activity lost from the blood to the activity gained by the tissues we have:

$$(1) \quad TA = VP_0(UA_0 - UA_k) + (VP_0 - V_k)(UA_k - UA_{tr}) + (VP_0 - V_k + V_{tr})(UA_z - UA_t)$$

where

TA = Total radioactivity in the extravascular component of tissue

VP<sub>0</sub> = Plasma volume at zero time

V<sub>k</sub> = Plasma volume of withdrawn blood

V<sub>tr</sub> = Volume of transfusion (plasma or saline)

UA<sub>0</sub> = Unit activity of plasma at zero time

UA<sub>k</sub> = Unit activity of plasma just before hemorrhage

UA<sub>tr</sub> = Unit activity of plasma just before transfusion

UA<sub>z</sub> = Unit activity of plasma just after transfusion

UA<sub>t</sub> = Unit activity of plasma at time of death

Following a transfusion of non-radioactive plasma or saline, the total radioactivity circulating before transfusion may be equated to the total radioactivity circulating after transfusion as follows:

$$(2) \quad (VP_0 - V_k)UA_{tr} = (VP_0 - V_k + V_{tr})UA_z$$

We have by substitution in (1),

$$(3) \quad TA = VP_0(UA_0 - UA_t) - V_k(UA_k - UA_t) - V_{tr}(UA_t)$$

or

$$(4) \quad TA = VP_0UA_0 - V_kUA_k - (VP_0 - V_k + V_{tr})UA_t$$

where

VP<sub>0</sub>UA<sub>0</sub> = Total radioactivity injected (This value does not depend on a plasma volume determination)

V<sub>k</sub>UA<sub>k</sub> = Total radioactivity removed by hemorrhage

(VP<sub>0</sub> - V<sub>k</sub> + V<sub>tr</sub>)UA<sub>t</sub> = Total radioactivity<sup>3</sup> left in the circulation at the time of death.

Where no transfusion is given V<sub>tr</sub> = 0. If F is the fraction of extravascular radioactivity per gram of dog (assuming the radioactivity lost from the blood to be evenly distributed throughout the animal) found in 1 gram of a

particular tissue, then

$$(5) \quad F = \frac{A}{\frac{TA}{W}}$$

where

A = The observed radioactivity of 1 gram of tissue

TA = Total radioactivity in the extravascular component of tissue (see equation (4))

W = Body Weight.

It follows that

$$(6) \quad \frac{F_s}{F_c} = \frac{A_s}{A_c} \times \frac{W_s}{W_c} \times \frac{TA_c}{TA_s}$$

when the subscript c represents the control dog and s represents the shocked dog. Now  $\frac{A_s}{A_c}$  is the "actual" ratio

given in Table II. The "corrected" ratio  $\frac{F_s}{F_c}$  is obtained by multiplying the "actual" ratio by the "corrected factor,"  $\frac{W_s}{W_c} \times \frac{TA_c}{TA_s}$  (Table II).

## RESULTS

The data of the individual experiments are shown in Figures 2 to 8, with accompanying legends. Table I summarizes protocol data which may be used as a reference for the interpretation of Table II, which gives the data in regard to plasma protein loss into various tissues.

## COMMENTS

From the figures of the foregoing experiments, it may be stated that the radioactivity curves of the circulating plasma in normal and shocked dogs, which have not received infusions, are substantially the same. The disappearance of radio-protein from the circulation, as judged by the

<sup>3</sup> Whenever available, a measured plasma volume was used instead of the theoretical volume (VP<sub>0</sub> - V<sub>k</sub> + V<sub>tr</sub>). When actual plasma volumes were not available, the estimated volume was used. This estimated volume we regard as reliable because the average difference between actual and expected plasma volumes in the early phase of shock is 9.3 per cent while in the late phase this difference is even less, except when infusions are given (See Table III).

<sup>2</sup> We are indebted to Professor Robley Evans, Massachusetts Institute of Technology, for deriving the following equations.

radioactive sulfur was injected before hemorrhage. Both control and shocked dogs suffered a nitritoid reaction with a drop in blood pressure to 70 mm. Hg. During the succeeding 6 hours, both blood pressures returned to normal. The shocked dog was then bled and shock ensued for 3 hours with a gradual decline of blood pressure to 45 mm. Hg. Blood withdrawal for plasma volume determination killed the shocked dog.

Six hours after the injection of radioactive plasma, the per cent of radioactive protein circulating in the plasma and the unit activity of plasma was about the same in both dogs. The slopes of the curves remained parallel during the onset of shock in the shocked dog. These observations are similar to those noted with radioactive bromoprotein (Figs. 2 and 3).



TABLE I  
Summary of protocol data

Experi- ment number	Figure number	Anesthesia in addition to morphine	Radioactive plasma			Plasma volume removed in hemor- rhage	Infusion	Duration of experiment after injec- tion of radioplasma	Dura- tion of shock	Plasma loss from analysis of tissue (see Table II)
			Bromine in bromoprotein	$\frac{W_c}{W_s} \times \frac{I_s}{I_c}$ *	Injection before or after hem- orrhage					
			<i>per cent</i>			<i>per cent</i>		<i>hours</i>	<i>hours</i>	
13 S <sub>1</sub>	2	Novocaine	1.0	1.0	Before	28	None	3	2	No loss Kidney, tho- racic skin Kidney Liver, ileum No loss Liver, skin, leg muscle
13 S <sub>2</sub>	2	Novocaine	1.0	1.0	Before	46	None	5	4	
11 S <sub>1</sub>	3	Novocaine	1.1	1.0	Before	45	None	7.5	4	
11 S <sub>2</sub>	3	Novocaine	1.1	1.0	Before	42	None	7.5	4.5	
7	4	Sod. barb.	0.1	0.91	After	31	None	14	11	
8 B	5	Sod. barb.	1.2	0.64	After	45	None	6.5	7	
9	6	Sod. barb.	0.1	0.87	Before	42	Plasma	14	9	
10		Sod. barb.	5.4	1.0	Before	15	Plasma	8	4.5	
12 S <sub>1</sub>		Sod. barb.	0.3	1.0	Before	30	Plasma	9	6	
12 S <sub>2</sub>		Sod. barb.	0.3	1.0	Before	43	Plasma	10	7	
8 A	7	Sod. barb.	1.2	0.84	After	68	Saline	4	4	Liver, lung, skin, leg muscle
S-1	8	Sod. barb.	Radiosulph- protein	0.76	After	39	Blood	9	10	
S-3		Sod. barb.	Radiosulph- protein	0.94	Before	25	Plasma	9.5	3	

\* This factor is the ratio of the proportion of plasma given to body weight of shocked dog to control dog, when W = wt. of dog, I = volume of radioactive plasma injected, c = control, dog and s = shocked dog.

curves of unit or residual radioactivity, occurs at approximately the same rate in the shocked and normal dog receiving the same specimens of radioactive bromoplasma. The unit radioactivity curves provide evidence as to whether or not a selective loss of protein or a shift in total water occurs in shock. The residual radioactive protein curves provide evidence as to whether or not whole plasma is lost in shock. But the validity of this latter curve depends on the validity of plasma volume determinations. Since there is doubt as to the reliability of plasma volume determinations under all circumstances, the conclusion from the slopes of the residual radioactive protein curves as to plasma loss into tissues is hazardous without a simultaneous comparison of the tissue content of radioactive protein in the normal and shocked dog. Such tissue analyses are useful not only for detecting plasma loss, but also for locating the area into which loss takes place.

Results of tissue analyses

Table II includes all measurements of radio-bromoprotein in tissues, expressed as the ratio of

the radioactivity of one gram of a given tissue in the shocked dog to that of the control dog. The actual ratio is corrected, as previously pointed out, to account for differences between these dogs in weight, in unit radioactivity of plasma, and in the total volume of circulating radioactive protein.

A corrected ratio of 100 per cent indicates equal escape of radiobromoprotein in the shocked and control dog. Assuming the error of the analytical technique to be some 5 to 25 per cent,<sup>4</sup> a corrected ratio of over 125 may be taken to indicate an excess of radiobromoprotein in the tissue of the shocked dog over that in the same tissue in the normal dog. Corrections for blood content of tissues were also made, in some experiments, in kidney, lung, and liver, in which the radioactivity due to blood varied from 2½ to 7½ per cent, 2 to 30 per cent, and 1 to 9 per cent, respectively.

A corrected ratio in excess of 125 may be taken to indicate loss from the circulation of whole plasma because the loss of circulating protein

<sup>4</sup> The range of error in the oxidation technique and measurement of radioactivity with the electroscope is 5 to 25 per cent, as shown in Table II and III of a foregoing publication in this series (5).

TABLE II  
Ratio of tissue radioactivity of shocked dog to control dog

Tissue	Experiment 13 (St)		Experiment 11 (St)		Experiment 11 (St)		Experiment 7		Experiment 8B		Experiment 9		Experiment 8A		Relative amounts of radioactivity in various tissues of 5 control dogs per gram
	Actual	Corrected	Actual	Corrected	Actual	Corrected	Actual	Corrected	Actual	Corrected	Actual	Corrected	Actual	Corrected	
Liver	75	95	45	59	80	99	180	150	74	77	120	160	170	185	5.5
Lung	60	76	83	110	73	91	140	120	8	8.5	51	71	150	165	6.6
Kidney	68	86	140	185	100	125	165	140	39	41	77	110	120	130	8.5
Stomach	53	67	51	67	67	83									3.5
Ileum	37	47	50	66	55	68	250	210	85	89	90	125	91	99	4.1
Colon	90	115	51	67	45	56									3.5
Thyroid	66	83	165	215	190	235	49	42	51	53			140	150	3.1
Skin (Thorax)	64	81	100	130	87	110	67	57	83	86	160	220	210	230	2.3
Skin (Back)	100	125	100	130	110	135	190	160	79	82			95	100	2.0
Heart	75	95	83	110	71	88	24	20	110	115	125	175	180	195	3.2
Muscle (Foreleg)	53	67	68	89	71	88	51	43	87	104	93	139	85	93	1.0
Muscle (Spine)	100	126	93	131	85	124	130	85					150	109	1.3
Plasma															7.7
Correction factor															

The ratios are corrected for radioactivity of blood content of tissue in experiments 13, 11, 9, in liver, lung, and kidney. Blood content of stomach, ileum, colon, skin, and muscle too small to be significant.

The "corrected" columns are calculated according to formulas given in text on page 297. This correction is necessitated by differences between normal and shocked dogs in total and unit radioactivity of circulating plasma. See Table I for protocol data.

without concomitant loss of the equivalent non-protein fraction would result in a fall in plasma protein concentration, while a loss of the non-protein fraction without loss of the equivalent protein fraction would cause a rise in the plasma protein concentration. Since the plasma protein concentration does not vary after the initial hemorrhage, except when infusions are given in late shock, protein loss is in proportion to whole plasma loss. This loss cannot be quantitated accurately because the unit activity of the lost plasma is not precisely known.

The ratios found in Experiments 13, 11 ( $S_1$ ), 11 ( $S_2$ ), 7, and 8B show no material difference between shocked and normal dogs in the amount of radioactive protein which has entered tissues from the circulation.

Where the ratios in certain exceptional instances are high, special analysis of their quantitative significance is necessary in organs whose weight is a substantial fraction of the total body weight. These include skeletal muscle, skin, liver, and gastro-intestinal tract. In skeletal muscle, no high ratio was found. In skin, a high ratio was found in 2 of 10, in liver in 1 of 5, and in gastro-intestinal tissue in 1 of 9 determinations.

In Experiments 13 and 8B, no loss of significance was observed in any tissue. In Experiment 11 ( $S_1$ ), skin of thorax showed a loss. If the skin of thorax is assumed to be some 10 per cent of the whole skin, the amount lost into it was calculated<sup>5</sup> as 45 cc. In this experiment, total loss is also significant. In Experiment 11 ( $S_2$ ), the same conclusion results, since the only loss was 60 cc. into the skin of the thorax. In Experiment 7, liver showed a loss of 35 cc. It is in this experiment that bowel showed a loss but we do not regard it as significant because it is the only instance in all our experiments, in which loss into bowel was observed.

Although the extent of plasma loss in these

<sup>5</sup> This calculation assumes that the plasma lost into the organ has the unit activity of the circulating plasma measured at the end of the experiment. The difference between the control and shocked dogs in corrected tissue activity, multiplied by the organ weight, and divided by this unit activity of plasma, provides the figure used. This calculation is of course only an approximate figure and weights the error in the direction of greatest loss.

experiments cannot be accurately assessed, the data on the whole indicate that such loss is small, that it involves only one or two tissues, which vary from one experiment to another, and that it is probably not quantitatively significant except when infusions are given in the late shock phase. Indeed the frequency with which one observes (in Table II) a substantially smaller content of radioactive plasma in the tissue of the shocked as compared to the control dog suggests that the reverse may be true, *i.e.* that less plasma moves out of the circulation in the shocked dog, presumably because of the contracted volume and decreased velocity of flow in the general capillary bed (10).

That plasma loss of perhaps significant proportions may occur when infusions are given in irreversible shock is evident from the data of Experiments 9 and 8A. A plasma infusion in the irreversible phase was given in Experiment 9 and a saline infusion in Experiment 8A. The skin and liver showed a high ratio in the shocked dogs of these experiments. Two of the three muscle determinations made showed high ratios. The only instance in which a high value for lung was observed was in Experiment 8A. The quantitative significance of the total loss in Experiment 8A (saline infusion) is probably substantial (see Legend to Figure 7). It is pertinent to refer here to the observation of Werle, Cosby, and Wiggers (11) that the exposed mucous membrane of the intestine in hemorrhagic shock was pale and shrunken, and only became engorged or hemorrhagic after fluids were given intravenously in the late shock phase.

The continued fall in unit concentration of radioactivity without a simultaneous escape of whole plasma, observed in all the uninfused control and shocked dogs studied, signifies dilution or escape of radioactive protein from the circulation. Continuous dilution does not occur in the normal dog and while there is evidence of fluid mobilization early in shock, this is certainly not true later on. Since the unit activity fell at the same rate in the normal and the shocked dogs, the equal loss of radioactive protein in both therefore signifies that the normal mechanism for the escape of such protein from the circulation was operating in both.

Other evidence that whole plasma does not

TABLE III  
Plasma volume in hemorrhagic shock

Experiment number	Plasma withdrawn to produce shock	Plasma volume in shock								Remarks
		First measurement				Second measurement				
		Duration of shock	Expected	Found	Gain or loss	Duration of shock	Expected	Found	Gain or loss	
	per cent	hours	per cent	per cent	per cent	hours	per cent	per cent	per cent	
1	46	1½	54	76	+22	4½	73	69	-4	Bleeding continued during shock
2	46	1	54	67	+13					
3	63	1	37	55	+18					
4	60	1½	40	60	+20	4½	55	57	+2	
5	71	3	29	54	+24					
6	35	2	65	46	-19					2nd reading 1½ hrs. after blood transfusion 2nd reading 3 hrs. after blood transfusion. Recovered. 2nd reading ¾ hr. after plasma transfusion. 2nd reading ½ hr. after plasma transfusion 2nd reading ½ hr. after 500 cc. saline infusion
7	36	3	64	64	0					
8	45	½	55	63	+8	3	56	56	0	
9	44	2½	56	72	+16					
10	28	3½	72	66	-6	4½	63	66	+3	
11	45	1	55	65	+10					
12	39	10	61	65	+4					
13	34	6	66	64	-2					
14	61	5½	39	75	+36					
15	40	2½	60	81	+21	4	61	65	+4	
16	25	1½	75	96	+21	3	85	85	0	
17	20	1	80	84	+4					
18	42	3	58	78	+20	4½	71	75	+4	
19	58	4	42	51	+9					
20	36	2½	64	53	-11	5	85	78	-6	
21	42	2½	58	64	+6	7	106	90	-16	
22	15	1	85	70	-15	2	123	98	-25	
23	42	3½	58	58	0	4	97	87	-10	
24	68	2	32	59	+27	2½	119	85	-34	
Average		+9.3								

All dogs died except as noted.

escape from the circulation in uninfused dogs in hemorrhagic shock is provided by the data in Table III,<sup>6</sup> showing plasma volumes in a group of shocked dogs. These volumes were determined by the method of Gibson and Evelyn (7).<sup>7</sup> All stages of shock were involved and it is evident

<sup>6</sup> This table, prepared by Dr. Howard Frank, includes data from a variety of experiments on shock, some of which have already been reported (Frank, H. A. and Fine, J., J. Clin. Invest., 1943, 22, 305, and Fine J., Fischmann, J., and Frank, H. A., Surgery, 1942, 12, 1.

<sup>7</sup> The error of the method was doubled in certain instances in which the galvanometer setting was 50 instead of 100. Such errors however would not affect the interpretation of the figures as presented.

(1) that there is no correlation between the volumes gained or lost and the duration or severity of shock; (2) only 4 of 24 dogs showed a loss, while 20 showed a gain—the average gain, 9.3 per cent. When plasma volume was measured again after a subsequent interval of shock (12 experiments), there was no significant gain or loss in this interval in 7. An average loss of 19 per cent in the remaining 5 occurred, but all of these were instances in which a preceding saline, plasma, or blood infusion had been given in the late shock phase, and the expected volume included the volume of the infusion. Whether the discrepancy between the expected and in-

without concomitant loss of the equivalent non-protein fraction would result in a fall in plasma protein concentration, while a loss of the non-protein fraction without loss of the equivalent protein fraction would cause a rise in the plasma protein concentration. Since the plasma protein concentration does not vary after the initial hemorrhage, except when infusions are given in late shock, protein loss is in proportion to whole plasma loss. This loss cannot be quantitated accurately because the unit activity of the lost plasma is not precisely known.

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The continued fall in unit concentration of radioactivity without a simultaneous escape of whole plasma, observed in all the uninfused control and shocked dogs studied, signifies dilution or escape of radioactive protein from the circulation. Continuous dilution does not occur in the normal dog and while there is evidence of fluid mobilization early in shock, this is certainly not true later on. Since the unit activity fell at the same rate in the normal and the shocked dogs, the equal loss of radioactive protein in both therefore signifies that the normal mechanism for the escape of such protein from the circulation was operating in both.

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3	63	1	37	55	+18					
4	60	1½	40	60	+20	4½	55	57	+2	
5	71	3	29	54	+24					
6	35	2	65	46	-19					
7	36	3	64	64	0					
8	45	½	55	63	+8	3	56	56	0	
9	44	2½	56	72	+16					
10	28	3½	72	66	-6	4½	63	66	+3	
11	45	1	55	65	+10					
12	39	10	61	65	+4					
13	34	6	66	64	-2					
14	61	5½	39	75	+36					
15	40	2½	60	81	+21	4	61	65	+4	
16	25	1½	75	96	+21	3	85	85	0	2nd reading 1½ hrs. after blood transfusion 2nd reading 3 hrs. after blood transfusion. Re-covered. 2nd reading ¾ hr. after plasma transfusion. 2nd reading ½ hr. after plasma transfusion 2nd reading ½ hr. after 500 cc. saline infusion
17	20	1	80	84	+4					
18	42	3	58	78	+20	4½	71	75	+4	
19	58	4	42	51	+9					
20	36	2½	64	53	-11	5	85	78	-6	
21	42	2½	58	64	+6	7	106	90	-16	
22	15	1	85	70	-15	2	123	98	-25	
23	42	3½	58	58	0	4	97	87	-10	
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Average					+9.3					

All dogs died except as noted.

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(1) that there is no correlation between the volumes gained or lost and the duration or severity of shock; (2) only 4 of 24 dogs showed a loss, while 20 showed a gain—the average gain, 9.3 per cent. When plasma volume was measured again after a subsequent interval of shock (12 experiments), there was no significant gain or loss in this interval in 7. An average loss of 19 per cent in the remaining 5 occurred, but all of these were instances in which a preceding saline, plasma, or blood infusion had been given in the late shock phase, and the expected volume included the volume of the infusion. Whether the discrepancy between the expected and in-

creased volumes in these 5 instances represents a true loss into tissues, or inadequate mixing of dye with capillary blood, is not clear and remains to be determined.

Our conclusion is that plasma volume does not fall beyond that accounted for by the amount withdrawn. This conclusion however rests upon the validity of the dye method. Since the rate of escape of dye from the blood stream during the period of measurement is corrected for by extrapolation of the disappearance curve, the two factors that may upset a true dilution figure are incomplete mixing with the peripheral stream or a measurement of dilution before complete mixing occurs. Although peripheral stagnation exists in shock, this is not equivalent to complete stasis. Complete mixing is therefore merely a function of time. The period of time for complete mixing is not known. In our experiments, three readings were made in a period of 20 to 35 minutes after dye injection. Assuming incomplete mixing, the result would be a false one, but in the direction of loss rather than gain because of the smaller volume involved if the mixing is incomplete. False values, which would be most likely in late shock, would therefore overestimate loss. Since our figures are already in favor of no loss, error in the method would only lend further strength to our conclusion. The results with respect to plasma loss obtained by the dye volume method therefore agree with those obtained by the radioactive protein technique.

#### DISCUSSION

Since the evidence is against a significant loss of plasma, from plasma volume studies and from the experiments with radioactive protein in which no infusions were given, the conclusion follows that death in hemorrhagic shock is not due to a *progressive* decline in plasma volume. It appears therefore that the changes induced by the *initial* decline in plasma volume are the cause of death and that the progressive disorder in hemodynamic equilibrium does not arise from an increasing imbalance in the fluid exchange between blood and tissues. The continuing local loss in burns or in areas of trauma do indeed constitute a progressive decline in plasma vol-

ume, but the difference between these states and hemorrhagic shock where hemorrhage has stopped may be merely a quantitative rather than a qualitative one. Toxic factors may also act deleteriously in burns or trauma, but as the mechanisms causing death in hemorrhagic shock seem not to involve capillary leakage, death from burns or trauma need not do so either. For, once the latter have produced a sufficient initial decline in plasma volume, the fatal mechanism is in operation without postulating still further decline as a necessary part of the fatal mechanism. This is an important consideration because even if one could correct a generalized increase in capillary permeability, death from burns or trauma would still result from the initial decline alone, if not corrected early enough. Therein lies the crux of the matter. For while the associated phenomena in different types of shock modify the pattern of events impressively, beneath them a mechanism exists, which in itself has fatal potentialities and which can produce all the basic phenomena common to all types of shock—namely, peripheral stagnation, lowered systolic and diastolic pressure, lowered venous oxygen, lowered venous pressure, decreased venous return, decreased cardiac output, increase in acid metabolites, and death.

#### SUMMARY AND CONCLUSIONS

A method is described for preparing radioactive bromoprotein which when infused into normal dogs behaves not unlike undenatured plasma protein (*e.g.* protein containing radioactive sulfur).

A technique of preparing tissue and plasma for radioactivity determinations is also described.

Radioactive proteins were given intravenously to normal dogs and dogs in hemorrhagic shock, both with and without anesthesia. Control dogs received the same radioactive protein given to the shocked dog in any single experiment. Disappearance curves of this radioactive protein from the circulation were obtained and plotted in terms of unit radioactivity and residual circulating radioactive protein. From these curves, the evidence was derived that the radioactive proteins disappear from the circulation at the same rate in normal and shocked dogs and that the

dog in shock from hemorrhage does not lose plasma into tissues. Tissue analyses of radioactive protein in these dogs gave the same result.

However, when the shocked dogs received intravenous infusions in the late or irreversible phase of shock, the radioactivity content of some tissues showed that plasma protein was lost into some tissues in possibly significant quantity. Similar evidence was obtained from plasma volume studies which showed that the deficiency in circulating plasma volume, created by the hemorrhage performed to induce shock, did not increase as shock progressed. If, however, infusions were given in the late shock phase, the expected volume was greater than the volume found. The data at hand were insufficient to determine whether the diminished volumes when found were due to actual leakage or inadequate mixing.

While the integrity of the capillaries may be impaired in the late shock phase, there is no evidence of a significant loss of plasma into tissues in untreated fatal shock following hemorrhage. Hence, an increase in capillary permeability is not a factor in the fatal issue. The phenomena which are set in motion by the initial critical loss in circulating plasma volume and which lead to death do not require that a progressive decline in plasma volume take place. Plasma loss into tissues therefore is not a crucial factor in hemorrhagic shock.

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# TRAUMATIC SHOCK. V. A STUDY OF THE EFFECT OF OXYGEN ON HEMORRHAGIC SHOCK

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The accumulating evidence from our studies of the plasma volume in hemorrhagic shock agrees with that of Gregersen (1) and of Evans (2), to the effect that there is no further loss of plasma from the circulation after the initial loss caused by the bleeding. Radioactive plasma proteins in the tissues of the dog in hemorrhagic shock are not found in greater concentration than in the unshocked dog. The irreversibility of hemorrhagic shock therefore cannot be explained by the theory of increased capillary permeability.

Venous anoxemia is one of the outspoken pathological phenomena in shock. It appears early, and the associated tissue anoxia, if prolonged sufficiently, might be considered responsible for the development of irreversibility because of irretrievable damage to vital structures. In that case, the prevention or amelioration of venous anoxemia might exert a beneficial therapeutic effect. Indeed, several recent reports (3, 4) claim such an effect from the administration of pure oxygen. The data given are not convincing, because the anoxemia was only partially corrected and the benefit derived consisted of temporary elevation of blood pressure or a prolongation of survival time by a few hours. A more definitive judgment as to the value of oxygen and the importance of venous anoxemia would require the prevention or complete correction of venous anoxemia. Only if a resulting change from the usual trend of events in shock is observed, can a therapeutic benefit from oxygen be claimed.

This communication presents experimental data showing that the usual trend of events in hemorrhagic shock is in no way influenced by oxygen so given as to prevent the occurrence of or restore to normal an already lowered venous oxygen.

## METHOD

Since pure oxygen by tracheal cannula given to a dog in shock usually fails to restore the venous oxygen to a normal level, oxygen at more than one atmosphere was

utilized with the expectation of better saturation, primarily because of increased oxygen in physical solution in the circulating plasma (5). Because four atmospheres of oxygen is lethal for dogs if carried on for the time intervals required for shock experiments, we performed all our experiments at three atmospheres of oxygen, in a pressure chamber made available to us by Dr. Drinker at the Harvard School of Public Health. Temperature and humidity were controlled throughout the experiment in the chamber and decompression carried out so as to avoid any ill effects from "the bends." Two control experiments were performed to observe possible deleterious effects of breathing three atmospheres of oxygen for a period of three hours—the maximum interval utilized in this study—in dogs prepared according to the technique described below, except that no blood was withdrawn. The dogs showed no effect on the blood pressure or in any other respect and recovery without incident ensued.

Mongrel dogs were anesthetized according to Wiggers' technique (6), i.e., about one-half hour following an initial dose of morphine sulphate (3.0 mgm. per kgm.) subcutaneously, sodium barbital (175 mgm. per kgm.) was given intravenously, two thirds of the dose in one injection and only as much more of the remaining third as was necessary for minimum anesthesia, sufficient to permit tracheal cannulation and exposure of vessels. No further anesthetic was required, although the lid reflex was present and many dogs were on the verge of recovering consciousness in the later stages. Blood was withdrawn fractionally from the femoral artery over a period of one-half to 1½ hours, until the arterial blood pressure fell to 70 mm. Hg and did not rise within fifteen minutes after the last withdrawal. No further blood, except for sampling, was removed, regardless of any subsequent recovery of blood pressure. In all, some 30 to 40 per cent of the estimated total blood volume was withdrawn. Plasma volumes before and after bleeding were measured by the method of Gibson and Evelyn (7). Hematocrits were determined in Wintrobe tubes containing heparin or oxalate. The plasma proteins were determined by the falling drop method, using heparin as an anticoagulant. All specimens for plasma volume, hematocrit, and plasma protein determinations were taken from the femoral artery. The blood pressure was measured at intervals in a mercury manometer by needle puncture of the femoral artery. Tests of reversibility by intravenous fluids, blood, or plasma were occasionally performed. Venous blood was taken from the right heart through a glass tube inserted into the left external jugular

vein. Samples for blood gas measurement were collected under oil, using crystalline oxalate as an anticoagulant, and analyzed by the manometric method of Van Slyke and Neill (8). Oxygen, in excess of hemoglobin saturation, dissolved in plasma at elevated pressures, presumably escaped before analysis at atmospheric pressure. Plasma pH determinations were made by the colorimetric method of Hastings and Sendroy (9). Oxygen was inhaled from a calibrated spirometer via a tracheal cannula. Expiration led out to the room air, except when oxygen consumption was measured, in which case it was returned through soda-lime to the spirometer.

### RESULTS

The data are presented in two groups: (I) experiments in which the oxygen was given after the dog was in shock, and (II) experiments in which the oxygen was started before blood was withdrawn or before shock from hemorrhage occurred.

*Group I. Oxygen at three atmospheres, given after shock was established.* Five dogs were bled to a shock level of blood pressure (70 mm. Hg) and were allowed to remain in shock  $1\frac{1}{2}$  to  $2\frac{1}{2}$

hours. Oxygen at three atmospheres was then supplied for  $1\frac{1}{2}$  hours or longer. In two dogs, the venous oxygen concentration was only partially restored (Figure 1). In the remaining three animals, the venous oxygen concentration returned to its original normal level or to a level above normal (Figure 2). Nevertheless, all five dogs exhibited a progressive decline in blood pressure terminating in death. The average survival time was about four hours following the termination of the bleeding. Only one dog showed a partial recovery of blood pressure, but death followed rapidly after decompression.

In three of the dogs in terminal collapse after decompression, the rapid intravenous injection of a volume of saline, exceeding that of the blood withdrawn, resulted in an increase in the venous blood oxygen, but collapse and death followed shortly thereafter. The rise in venous oxygen in one instance (Figure 3) greatly exceeded that effected by oxygen inhalation. In a second instance, the already normal venous oxygen was

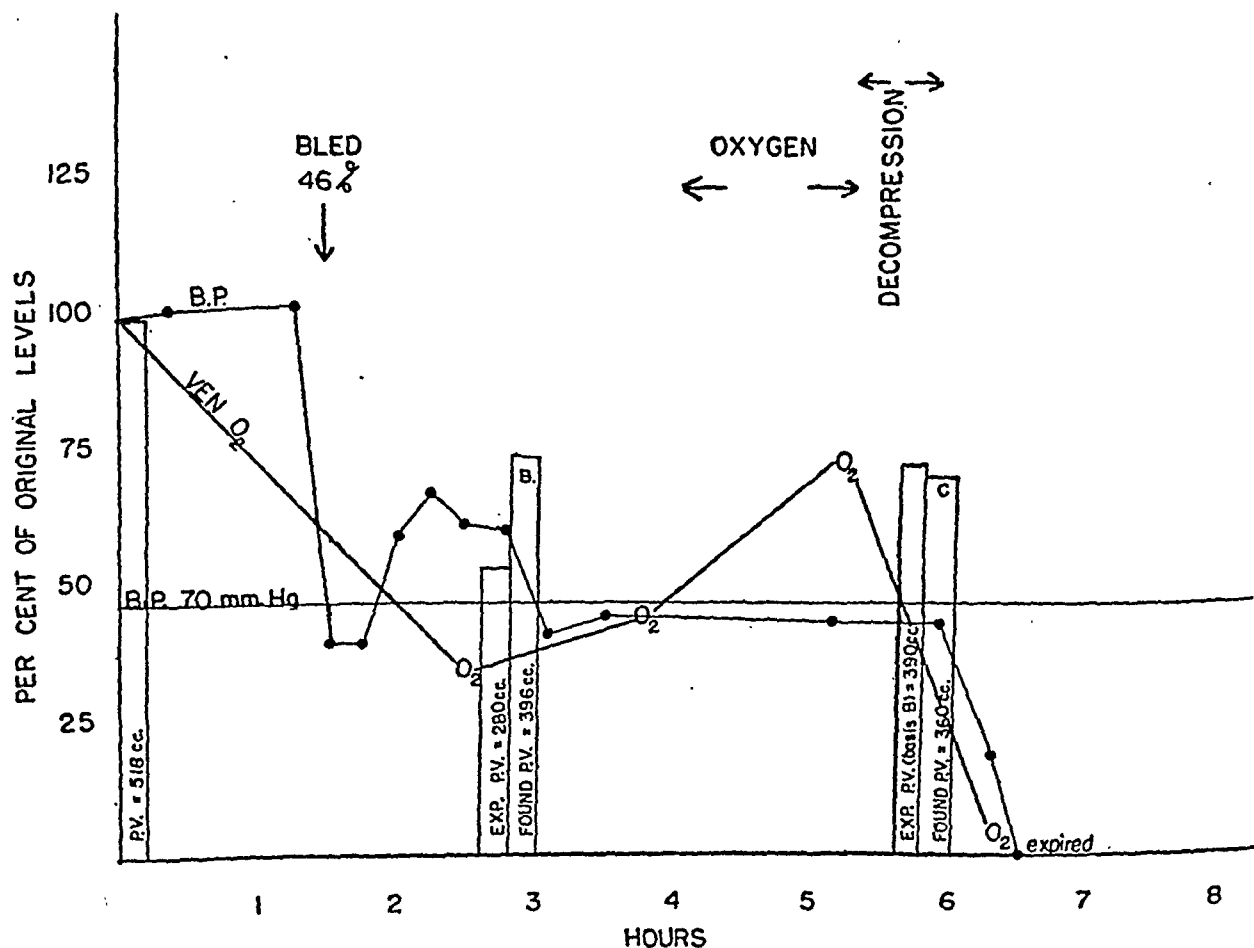


FIG. 1. (Exp. 2.) Slight mobilization of plasma after bleeding. No further loss during shock state. Oxygen inhalation corrected anoxemia without influencing blood pressure or prolonging life.

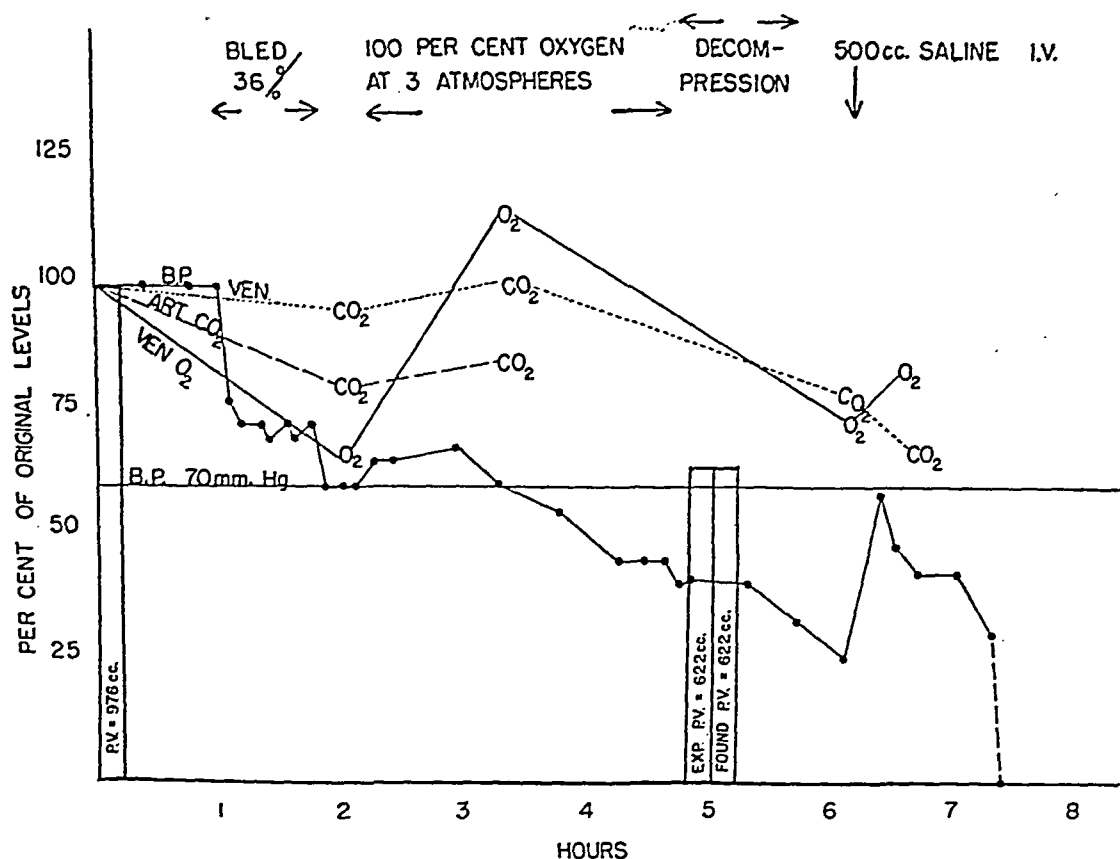


FIG. 2. (Exp. 11.) No plasma loss from capillary leakage. Anoxemia corrected but no effect on blood pressure or survival time.

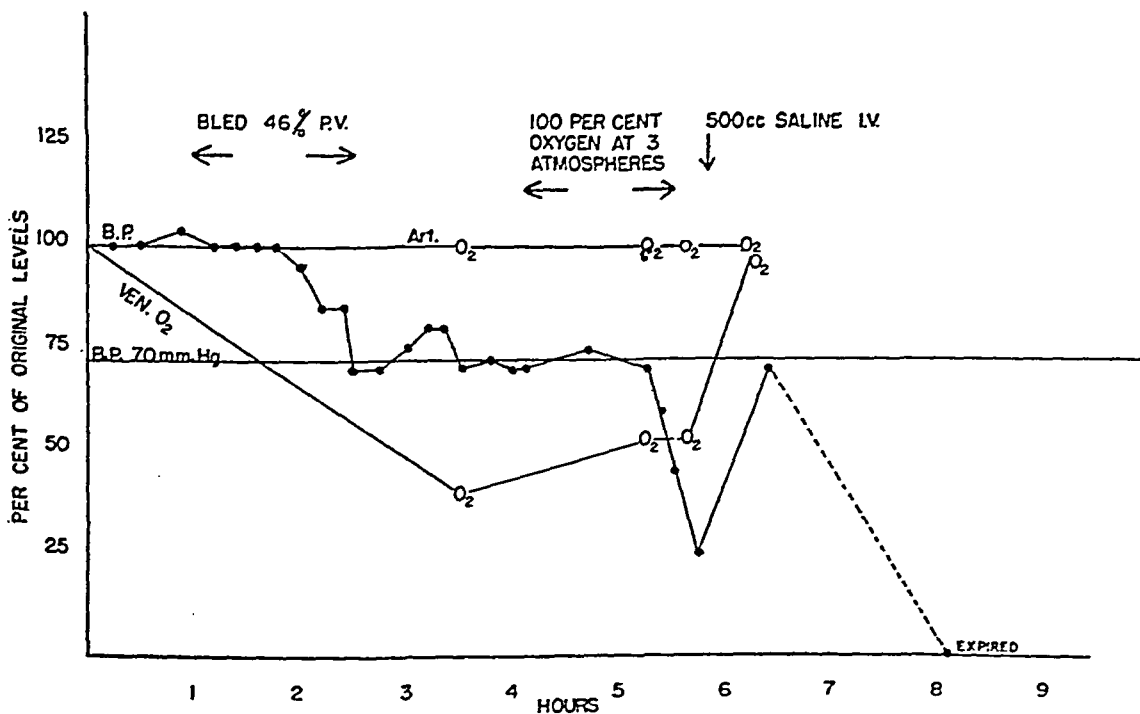


FIG. 3. (Exp. 4.) Inhalation of oxygen partially improved venous anoxemia but saline solution restored it to normal. Blood pressure and survival time not affected by oxygen.

TABLE I  
*Arterial and venous blood oxygen content before and during shock, and before and during the inhalation of oxygen at 3 atmospheres*

Control period				Shock period				Survival following onset shock
Experiment number	Arterial O <sub>2</sub>	Venous O <sub>2</sub>	Blood volume withdrawn	Breathing room air		Breathing oxygen at 3 atmospheres		
				Arterial O <sub>2</sub>	Venous O <sub>2</sub>	Arterial O <sub>2</sub>	Venous O <sub>2</sub>	
	volumes per cent		per cent	volumes per cent		volumes per cent		hours
4	19.0	15.0	46	19.1	5.9	19.0	8.1	3½
2	18.9	17.6	46	19.3	6.6		13.4	5
11		13.4	36		8.7		15.4	4½
5	17.8	14.8	63	18.9	8.3	20.9	15.0	3½
6	22.8	16.7	60	23.1	10.3		16.8	4½

increased, and in a third, a rapidly falling venous oxygen level was increased only slightly. The effect of intravenous fluids on the venous oxygen was regarded as a result of the temporarily increased velocity of flow.

A period of 1½ to three hours of inhalation of oxygen at three atmospheres therefore did not modify the course of events in shock from hemor-

rhage, whether the venous oxygen was or was not restored to normal (Table I).

*Group II. Oxygen at three atmospheres given prior to bleeding and continued during shock.* In an attempt to eliminate any period of anoxemia which might cause injury not amenable to subsequent oxygen therapy, five dogs were bled in the same manner as those in Group I, but after

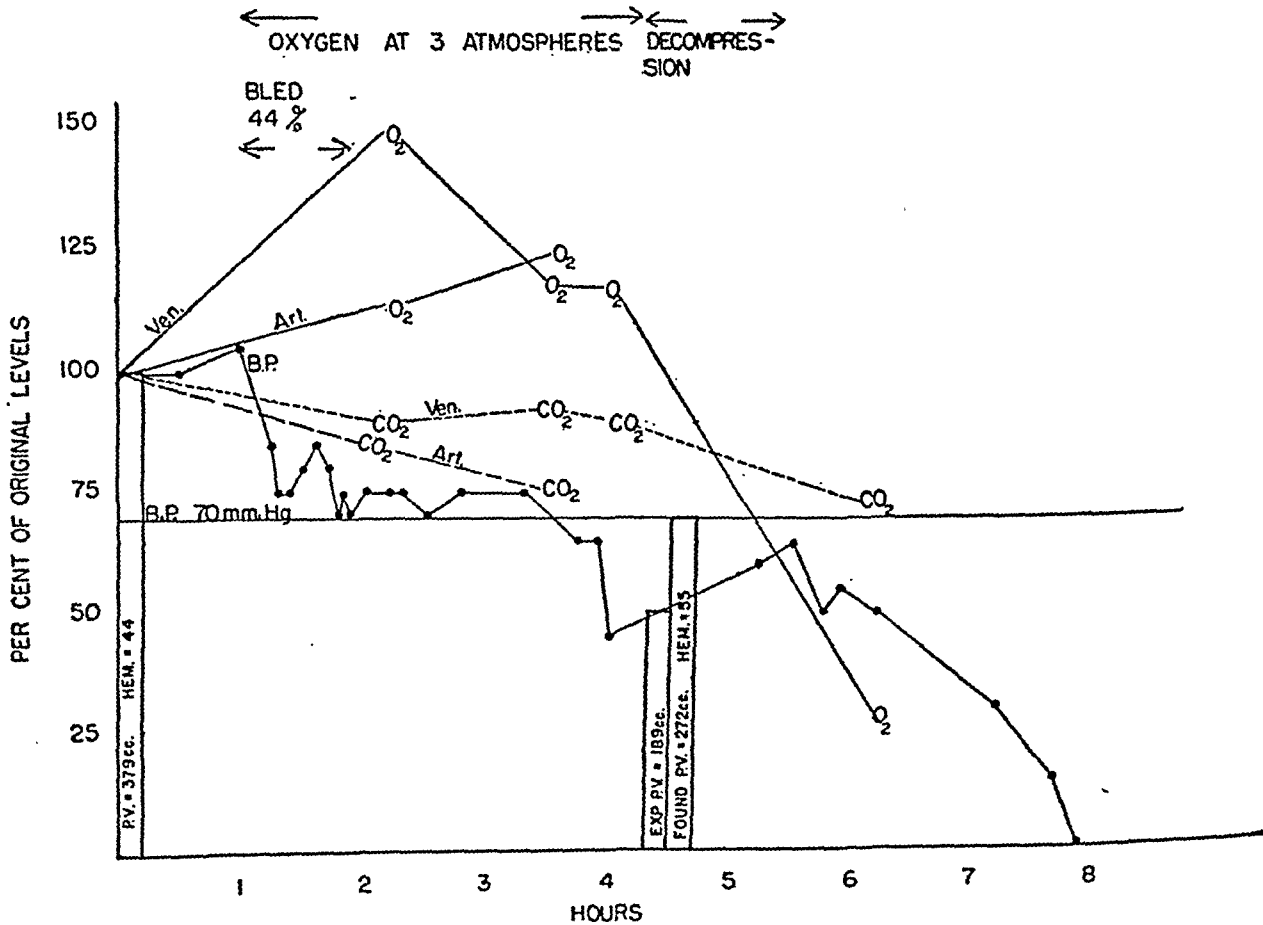


FIG. 4. (Exp. 15.) Plasma volume not lost into tissues during shock. Anoxemia prevented by oxygen inhalation but survival time and blood pressure not affected.

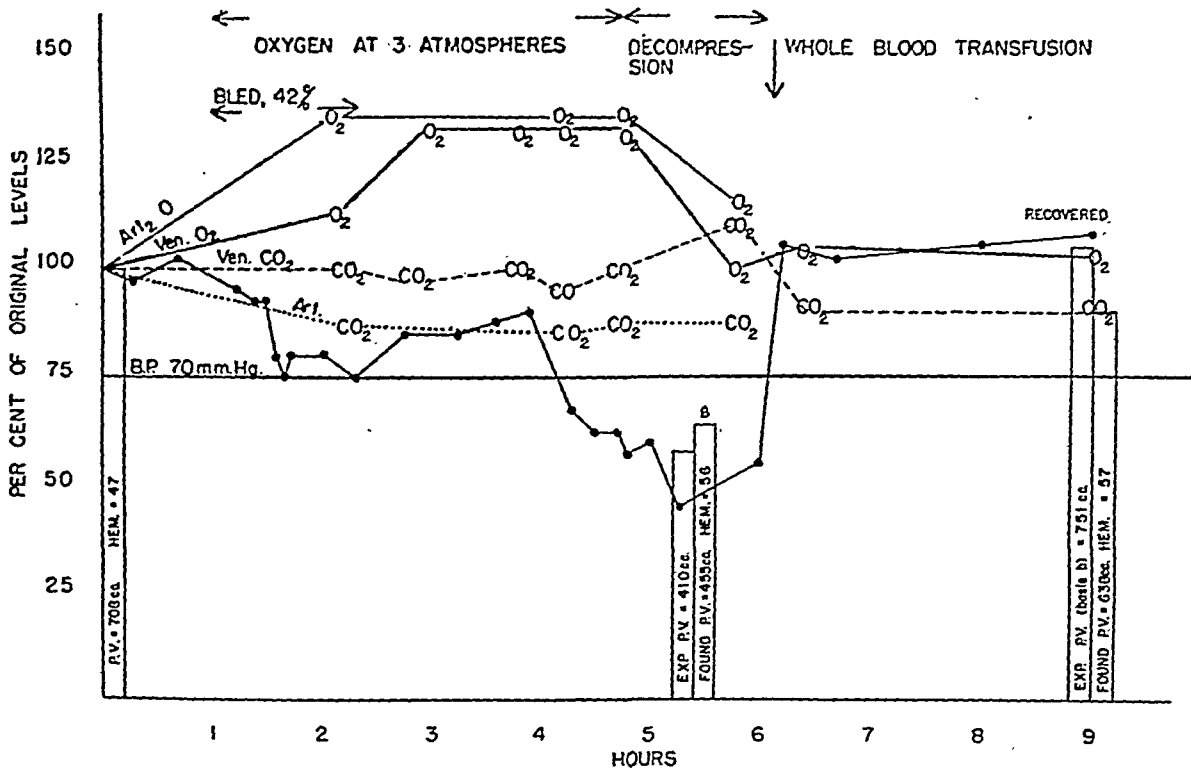


FIG. 5. (Exp. 14.) This dog began to fail while in oxygen and while venous oxygen was above normal. Transfusion caused recovery. Plasma volume did not escape into tissues while in shock phase.

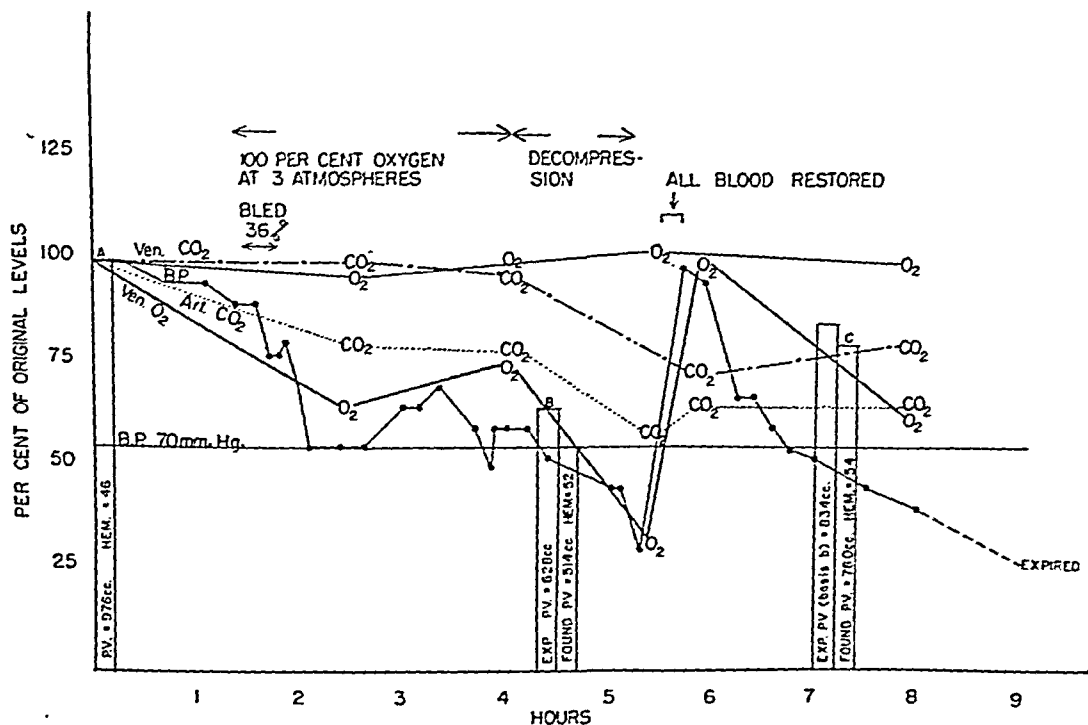


FIG. 6. (Exp. 13.) Venous anoxemia not prevented by oxygen inhalation. Irreversible shock developed in oxygen. Transfusion in irreversible phase corrected anoxemia, but only temporarily. Fatal decline after transfusion not accompanied by fall in plasma volume.



oxygen at three atmospheres had been started. The oxygen was continued for  $2\frac{1}{2}$  to three hours and the train of events observed. The course of circulatory collapse was in no way different from what we and many others have commonly observed when shock is induced by hemorrhage (Figure 4, Table II). The condition of the dog in every case became clearly terminal while oxygen was still being administered. All but one of the dogs in this group died. The surviving animal (Figure 5) recovered after a rapid re-injection of whole blood, equal in volume to that removed. In another dog (Figure 6), a similar procedure failed to produce more than a temporary recovery.

Table II shows that three atmospheres of oxygen given in advance of the onset of shock and continued during shock failed to sustain the venous oxygen concentration in five of ten experiments. In these dogs, collapse and death occurred much earlier (average survival time  $3\frac{3}{5}$  hours) than in the remaining five (average survival time  $6\frac{1}{2}$  hours). One cannot, however, infer a relationship between the speed of the collapse and the ineffectiveness of oxygen inhalation on the venous oxygen concentration, because the survival time of dogs in which the venous oxygen was maintained at normal or above normal levels was no longer than that of dogs in Table I in which the venous oxygen fell, or of other dogs in similar experiments of our own or of others in which no oxygen was given.

Those who have been particularly concerned with the establishment of criteria by which shock induced by hemorrhage may be said to exist, might question the validity of the foregoing experiments on the ground that these dogs may have succumbed to hemorrhage without developing shock. Three experiments (Figures 7, 8, 9) therefore were performed strictly according to the standard of irreversibility in shock from hemorrhage set up by Wiggers and Werle (10), i.e., sufficient bleeding to produce a hypotension of 50 mm. Hg for 90 minutes and 30 mm. Hg for an additional 45 minutes, following which blood transfusion, in their experience, uniformly failed to restore the animal. Oxygen at three atmospheres was started before the bleeding. All three dogs died while still in oxygen, two of them before blood transfusion could be given, and the third despite the return of all withdrawn blood.

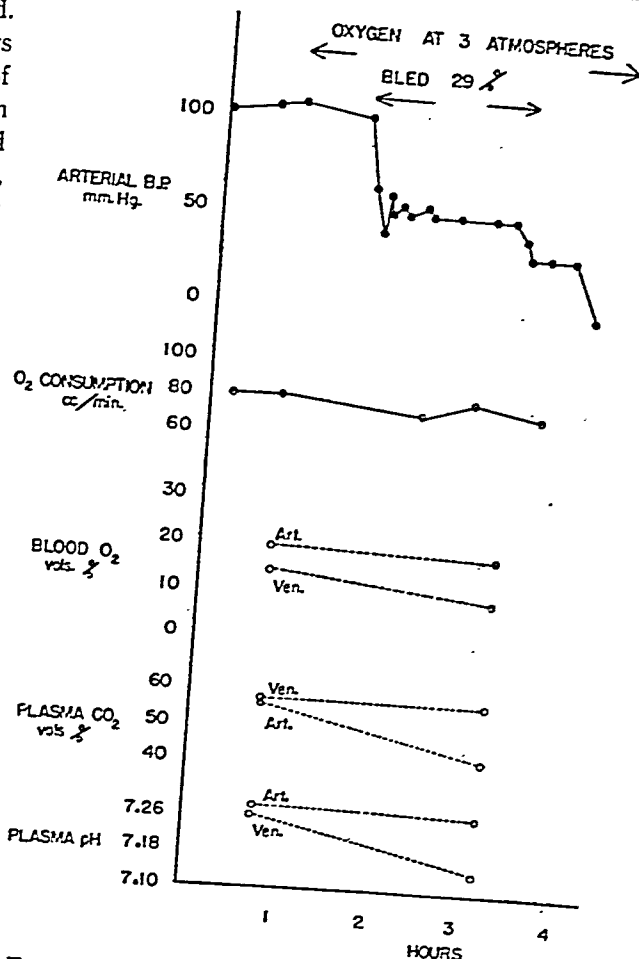


FIG. 7. (Exp. 22.) Death during inhalation of oxygen at three atmospheres despite the restoration of all blood withdrawn. Venous anoxemia not prevented but total oxygen consumption maintained. Increased acidity of venous blood in shock although carbon dioxide content diminished.

The blood pressure, venous and arterial oxygen and carbon dioxide and plasma pH curves are shown. The data are substantially the same as those of our previous experiments and those of Price *et al.* (11).

Since pure oxygen, even at three atmospheres, failed to alter favorably any of the commonly observed phenomena in shock, including the survival time, even when the venous oxygen concentration was maintained at normal levels (7 of 15 experiments), the likelihood of a beneficial effect from the employment of oxygen at one atmosphere as a therapeutic agent in hemorrhagic shock seems quite remote.



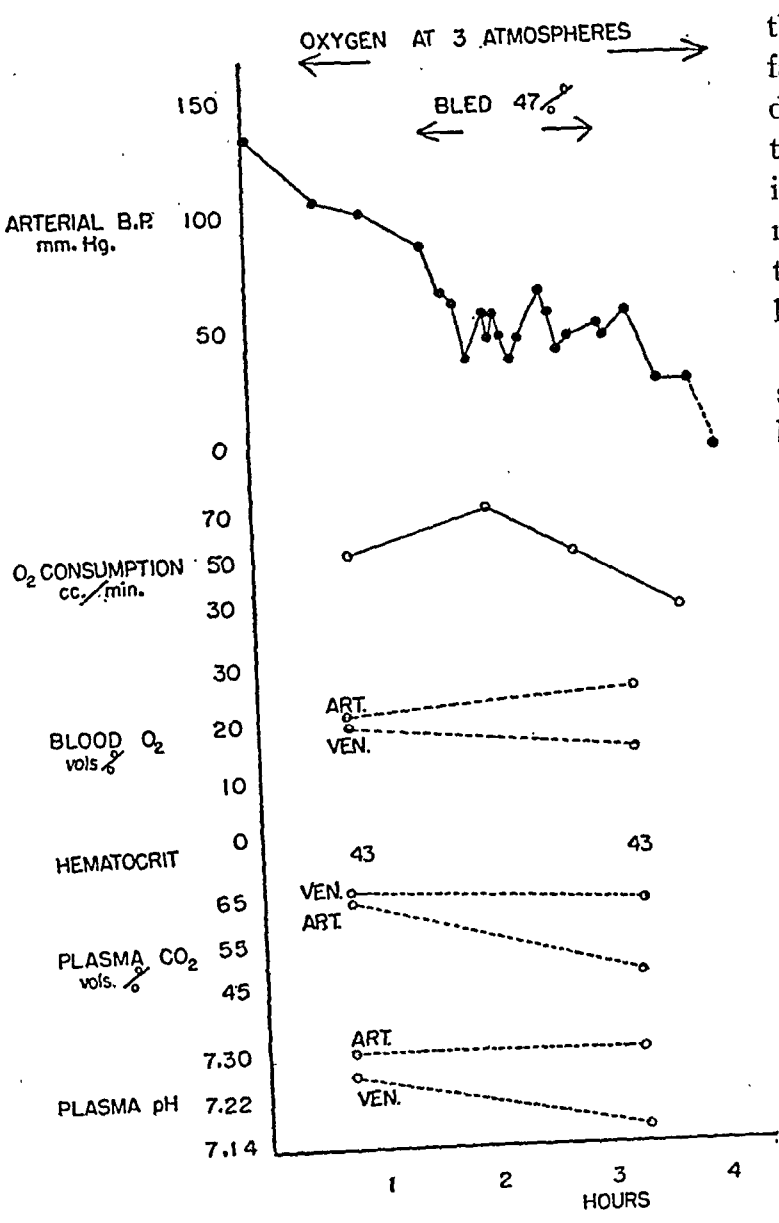


FIG. 8. (Exp. 23.) Death during inhalation of oxygen at three atmospheres. Moderate fall in venous oxygen content but total oxygen consumption not reduced until just before death. Venous blood more acid without change in carbon dioxide content.

Tissue anoxia is presumed to exist in shock because of the low venous oxygen concentration. It is possible, however, that tissue anoxia may continue in spite of the fact that venous oxygen has been restored to normal by the inhalation of oxygen at high pressure, (1) because of actual arterio-venous shunts, or (2) because of the existence of peripheral fields of circulatory failure, whose segregation from active circulation creates what may be regarded in effect as arterio-venous shunts. If tissue anoxia exists, oxygen consumption might fall, providing the tissue requirements have not increased. It is generally assumed that

these requirements are decreased, as shown by falling temperature, decreased activity of muscles, decreased urinary excretion, etc. To what extent these decreased requirements are offset by the increased demand of the respiratory muscles is not known. Aub (12) and Blalock (13) found that oxygen consumption did not fall until the late stage of hemorrhagic shock. Gregersen (14) found a substantial decrease in oxygen consumption during traumatic shock. In eight dogs breathing oxygen at three atmospheres, we noted

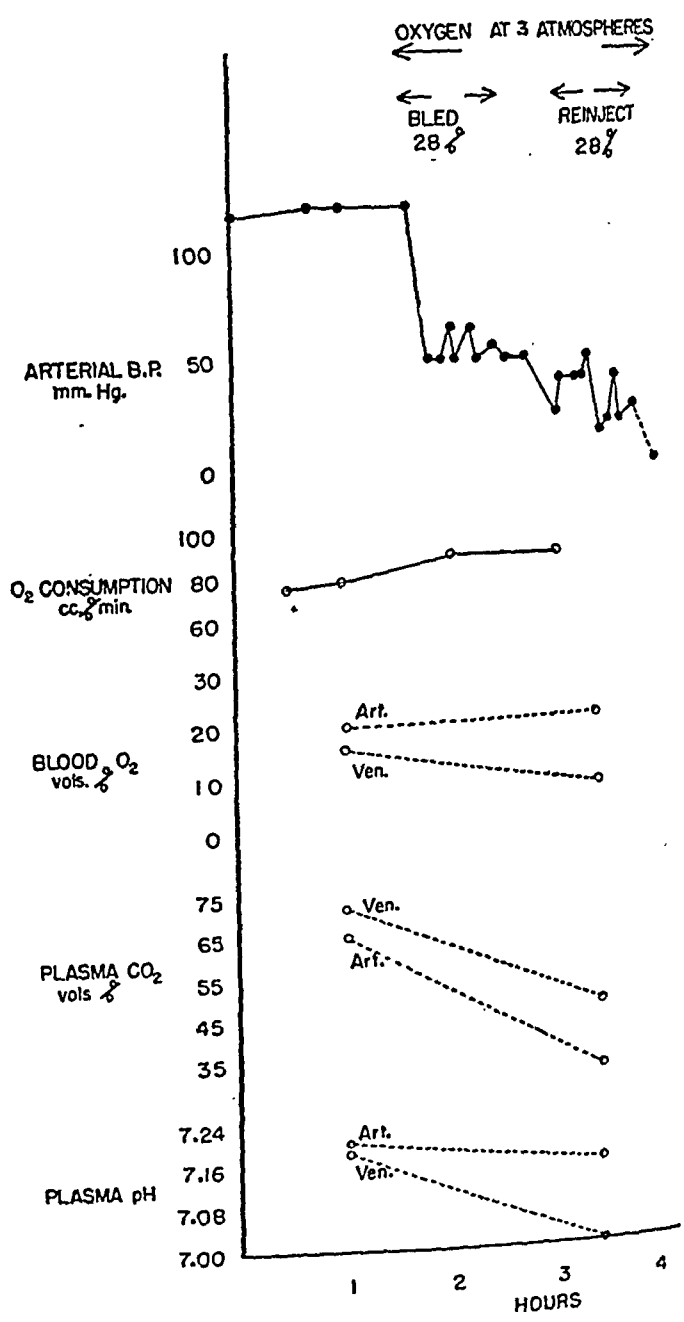


FIG. 9. (Exp. 24.) Death during inhalation of oxygen at three atmospheres. Maintained oxygen consumption and unchanged venous carbon dioxide content. Increased acidity of venous blood.

TABLE III

*Blood oxygen, carbon dioxide content, and total oxygen consumption before and after bleeding, by technique of Wiggers and Werle, to shock in oxygen at 3 atmospheres*

Experiment number	Control period					Blood volume with-drawn	Breathing O <sub>2</sub> in 3 atmospheres					Survival following onset shock
	Arterial		Venous		O <sub>2</sub> con-sumption		Arterial		Venous		O <sub>2</sub> con-sumption	
	O <sub>2</sub>	CO <sub>2</sub> *	O <sub>2</sub>	CO <sub>2</sub> *			O <sub>2</sub>	CO <sub>2</sub> *	O <sub>2</sub>	CO <sub>2</sub> *		
	volumes per cent		volumes per cent				cc. per minute	per cent	volumes per cent			
22	21.1	64.4	18.7	67.6	57	28	26.5	48.0	14.6	64.3	58	2½
23	18.8	65.0	15.8	72.8	75	28	20.8	33.6	8.6	48.0	91	2¼ (Transfusion)
24	19.0	58.1	14.1	58.3	80	29	18.0	42.2	9.1	57.1	78	2¼

\* Plasma analyses.

an increase in oxygen consumption during hemorrhagic shock until just before death. The increase was presumably due to the marked hyperpnea uniformly observed in such animals.

That the lowered venous oxygen, to some extent at least, is apparently merely an index of a decrease in the velocity of peripheral blood flow, is evident from the fact that temporarily increased velocity of flow in the late shock phase, following intravenous saline solution, causes a rise in venous oxygen. If this inference is correct, the carbon dioxide content of the venous blood should also vary as the velocity of flow. This is, in fact, the case. Studies of the carbon dioxide concentration on arterial and venous bloods in hemorrhagic shock show, as expected, a lowered arterial carbon dioxide concentration due to increased pulmonary ventilation. The venous carbon dioxide remains normal or falls, but in the latter case, not in proportion to the original difference between arterial and venous carbon dioxide. Furthermore, in three experiments following the infusion of saline solution or blood, the venous carbon dioxide content fell still further (Figures 2, 5, 6) although in one experiment, an observation just before death, no substantial change occurred.

The decreasing pH (Figures 7, 8, 9) and the increasing divergence in the A-V difference in pH as shock proceeds are evidence of an increase in the concentration of non-volatile metabolites. This may in part be due to the mechanisms governing the velocity of peripheral blood flow.

#### CONCLUSION

The course of events in hemorrhagic shock is in no way altered when venous anoxemia is pre-

vented by administering oxygen at high pressure. Tissue anoxia in these circumstances may still exist, presumably because peripheral stagnation results in what amounts to the existence of an arteriovenous shunt. Oxygen as a therapeutic agent in hemorrhagic shock is therefore of doubtful value.

Further evidence is given that the velocity of peripheral blood flow in hemorrhagic shock is reduced.

Acknowledgment is made to Drs. Arnold M. Seligman, Leroy D. Vandam, and Stanley M. Levenson for assistance in the work in the pressure chamber.

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# PRODUCTION OF TETANUS TOXIN ON PEPTONE-FREE MEDIA<sup>1</sup>

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An investigation of the growth requirements of *Cl. tetani* was undertaken in 1939, with the hope that through a knowledge of them, it might be possible to produce a uniformly potent toxin on a medium free from peptone, or any other material possessing antigenic effect of its own. Several reports in the literature had described acute anaphylactic-like symptoms following a second or third injection of tetanus toxoid for human prophylaxis, and in a few instances (1 to 3), analysis of the case showed that Witte's peptone, used in the original medium, was responsible.

Considerable progress has been made in the studies on nutritional factors (4). Parallel with these, work was commenced on toxin production, and it soon became evident that with this organism, as with *C. diphtheriae* (5), peptone was not essential and could be replaced by complete acid hydrolysates of protein, and presumably by suitable pure amino acids. Unexpectedly, it developed that the concentration of iron in the medium seemed to play the same crucial part as in diphtheria toxin production (6, 7). With suitable control of this factor, and in media containing the appropriate materials, all of relatively small molecular size and therefore not of themselves antigenic, toxin was obtained with regularity.

Preliminary reports of this work have already been made (8, 9). It is the purpose of the present communication to present, in somewhat greater detail, certain of the experimental aspects of these studies which will serve as a basis for attempts to place tetanus toxin production with simplified media on a practical basis.

## PRELIMINARY ASPECTS

The strain of *Cl. tetani* used in the New York State Division of Laboratories was selected by random choice.

<sup>1</sup> Aided by a grant from the Commonwealth Fund.

It is stated to produce toxin of 10,000 to 20,000 guinea pig MLD per cc. We have carried it in stock by the rather unorthodox method of serial daily transplant on ordinary meat infusion peptone broth containing 1.0 per cent glucose, grown in anaerobic jars (10) at 36° C. Probably as a result of this procedure, the ability to form spores seems to have been suppressed and they are not found, even in very old cultures. Toxin production seems to have been unaffected. There is a tradition among producers of tetanus toxin that an inoculum of spores, or at any rate of an old culture yields toxin of higher titer than does a young, actively growing culture. In our experiments, no such difference has been observed in occasional comparisons of our serial transfer strain with older ones.

Experimental media have been handled in two ways—either in 10 cc. amounts in 6 × 5½ inch test tubes, or in 125 cc. Erlenmeyer flasks. In the former case, the cotton plugged tubes have been autoclaved at 10 pounds pressure for from 7 to 10 minutes, promptly cooled, and inoculated. The centrifuged sediment from a 24-hour infusion broth culture is suspended in 1 or 2 cc. of sterile hydrolysate medium, and an inoculum of 0.05 to 0.1 cc. of the suspension is delivered to the bottom of each tube with a capillary. Incubation at 36° C. is carried out in an anaerobic jar. The flasked media have been autoclaved at 10 pounds for 15 minutes, the containers being about ¾ full and covered with 30 cc. glass beakers. Immediately upon removal from the autoclave, the flasks to be inoculated are filled to the neck from an extra supply, cooled in water, inoculated with a few drops of a suspension and incubated either anaerobically or in the air, at 36° C. Maximum toxin production is reached after 8 days, but in many experiments, toxin titrations have been done on the fourth or fifth day.

The estimation of toxin has been carried out in mice, using an inbred strain of white Swiss. The toxins to be examined are well mixed with one tenth volume of 5.0 per cent phenol, centrifuged for half an hour, and the supernatants are suitably diluted with 1.0 per cent Witte peptone in saline. The mice are injected subcutaneously along the back, the needle being inserted near the root of the tail, with 0.5 cc. quantities of the desired dilutions. By using 2 or 3 mice for each sample of toxin, a reasonable estimate of comparative potency may be obtained. A number of attempts to estimate the toxin by flocculation have shown no convincing parallelism between the titers obtained and toxicity for animals.

## COMPONENTS OF MEDIUM

The growth requirements of *Cl. tetani* have proved to be highly complex. The greater part of the early experiments on toxin formation were therefore carried out with relatively uncharacterized mixtures of nutrients. To a certain extent this has continued to be true because of practical difficulties. An acid hydrolysate of casein has been employed to supply amino acids, since it appeared to be the most convenient and least expensive source. Various fractions of liver extract were incorporated to furnish the necessary growth accessories. Eventually, most of the latter could be provided in pure form, but the requirements for the substance which has been named "folic acid," the constitution of which is still unknown, continues to necessitate the use of a concentrate.

There would be no object in recording the numerous experiments carried on over a period of 2 years in the attempt to arrive at uniformly maximum titers. The many chemical factors involved in growth require laborious repetitions of tests in which each individual factor, so far as possible, is varied against a constant of all the others. Only in this way can it be determined which concentrations are critical and must be carefully adjusted. Moreover, the concentration of Fe must be controlled as carefully as in the preparation of diphtheria toxin, and since the permissible level is so low as to be beyond simple and reliable chemical measurement, several tubes of each modification of medium within an experiment, each at a different iron concentration, must usually be made and tested.

It has been possible to obtain toxin of the same, or even a higher, degree of potency as that said to be produced by this strain in the New York State Laboratories (10,000 to 20,000 MLD's for guinea pigs). Efforts to obtain strikingly higher yields have thus far failed, and it is possible that the limit for the particular strain has been reached. An investigation of other cultures will be undertaken in due course. The following experiment describes the production of one of the better lots of toxin.

## Experiment March 13, 1941—p. 74.

Casein hydrolysate (15.4 mgm. N per cc.) <sup>2</sup> ..	240. cc.
Accessories and metals <sup>3</sup> .....	2.4 cc.
Cystine, 20 per cent in HCl.....	2.4 cc.
Tryptophane, 1 per cent.....	12. cc.
Glucose.....	12. grams
Calcium pantothenate.....	0.3 mgm.
Liver eluate <sup>4</sup> .....	9. cc.
Water to make.....	1200. cc.

After the various ingredients had been mixed and diluted to 1200 cc., the excess iron was removed by adding 6.0 cc. of 10 per cent  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , adjusting the reaction to pH 7.6, bringing to a boil, and filtering. The filtrate was cooled, and further iron removed by adding about 1.5 cc. calcium chloride and repeating the process.

The resulting medium was autoclaved in six 250 cc. Erlenmeyer flasks for 15 minutes, at 10 pounds pressure. Four of the flasks were then filled completely full from the other 2, and cooled promptly. They were inoculated as described earlier, and incubated in anaerobic jars at 36° C. for 5 days.

The resulting toxoid was centrifuged and filtered partly through a Berkefeld "N," the rest through a "V." The "V" filtrate was titrated in both mice and guinea pigs. The results follow:

*Mice* (Received 0.5 cc. of the stated dilution subcutaneously).

1 : 40,000.....	Dead 48 hours
1 : 80,000.....	Dead 64 hours
1 : 160,000.....	Slight symptoms 96 hours
1 : 320,000.....	Slight symptoms 96 hours
MLD > 160,000	

<sup>2</sup> A low chloride hydrochloric acid hydrolysate, prepared as described by Mueller and Johnson (11), from which excess Cl was removed by  $\text{PbO}$ . It contains sufficient  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$  to give a final concentration in the medium of about 0.1 per cent and 0.033 per cent respectively.

<sup>3</sup> Prepared as for diphtheria toxin (12) and contains  $\text{MgSO}_4$ , traces of Cu, Mn, and Zn, together with nicotinic acid, beta alanine, and pimelic acid.

<sup>4</sup> Prepared from the 90 per cent alcohol soluble fraction of liver extract, supplied us through the courtesy of Dr. Defries of the Connought Laboratories, University of Toronto. This was further purified by absorbing with norite charcoal, washing the latter first with water, then with hot 50 per cent alcohol, and then eluting with dilute pyridine-alcohol solution. After removal of the organic solvents *in vacuo*, the aqueous solution was preserved in the ice-box with toluol. One cc. was roughly equivalent to 62 grams of fresh liver.

Guinea Pigs (Received 1.0 cc. subcutaneously).

1 : 20,000.....	Dead 60 hours
1 : 30,000.....	Dead 62 hours
1 : 40,000.....	Dead 93 hours
1 : 50,000.....	Dead 93 hours
1 : 60,000.....	Dead 98 hours

MLD 50,000

The following experiment illustrates the marked inhibition of toxin formation by traces of iron salts.

Experiment May 2, 1941—p. 105.

Casein hydrolysate (17.0 mgm. N per cc.)..	18 cc.
Accessories and metals.....	0.2 cc.
Cystine, 20 per cent.....	0.2 cc.
Tryptophane, 1 per cent.....	1.0 cc.
Glucose.....	1.0 gram
Calcium pantothenate.....	0.025 mgm.
Liver eluate.....	0.75 cc.
Water to make.....	100. cc.

The iron was removed twice by precipitating together with calcium phosphate. Five tubes, of 15 cc. each, were prepared and the following quantities of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  were added: 0, 4, 8, 16, and 32 micrograms. The tubes were autoclaved, cooled, inoculated, and incubated anaerobically for 5 days. One and a half cc. of 5 per cent phenol was then mixed with the contents of each tube. After centrifuging for half an hour, the supernatants were diluted with 1 per cent Witte peptone in saline, and 0.5 cc. subcutaneous injections given a series of mice.

Toxin dilution	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}/15$ cc. medium				
	0	4 gamma	8 gamma	16 gamma	32 gamma
1 : 20,000	Dead 70 hours	D 90 hrs.	SS	S	0
1 : 40,000	Dead 70 hours	SSSS	SS	0	0
1 : 80,000	Dead 90 hours	SS	S	0	0
1 : 160,000	SSSS	S	0	0	0

Increased degrees of paralysis at 96 hours are indicated as S, SS, etc.

#### EFFECT OF OTHER INDIVIDUAL COMPONENTS

Variation, within reasonable limits, of the concentrations of the other ingredients of the medium appears to be without marked effect on toxin formation. Increase in the amount of liver extract concentrate, seems, on the whole, to be

unfavorable to toxin, whereas its reduction results in failure to obtain growth. The casein hydrolysate can be somewhat diminished without loss of toxin, while its increase retards toxin and growth, probably through osmotic effects and perhaps through excessive concentrations of certain amino acids.

Omission of traces of Cu, Mn, and Zn tends to lower the amount of toxin somewhat, but it has been difficult to obtain clear cut results. Nicotinic acid, beta-alanine, and pimelic acid are without effect, so far as it is possible to observe, and, if required, are evidently supplied by the liver concentrate in adequate quantity.

Cystine and glucose probably contribute to the production of anaerobic conditions in the freshly heated medium. Either one may be omitted with no impairment of growth or toxin. In the absence of both, growth fails.

Tryptophane also is required for multiplication of the organisms, as are the inorganic salts of K, Mg, and  $\text{PO}_4$ . The concentrations are not particularly critical.

It is clear that while using such complex mixtures as casein hydrolysate and the liver extract concentrate, the appraisal of optimal concentrations of many individual factors is impossible. When it becomes possible to substitute pure amino acids and growth accessories for these materials, progress in this direction may be expected, and, conceivably, higher yields of toxin may be obtained.

#### SUMMARY

The production of tetanus toxin on a medium free from peptone has been accomplished with one strain of *Cl. tetani*. As good, or somewhat better, toxin titers have been obtained as this strain (New York State Department of Laboratories) repeatedly yields on peptone-infusion media. The composition of the medium is very similar to that developed in this laboratory for diphtheria toxin, but is relatively more complex because of the greater number of growth factors demanded by the tetanus bacillus. As with diphtheria toxin, a very low and accurately controlled concentration of Fe is essential for optimal yields. Attempts to effect further simplifications and improvements in the medium are being continued.

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

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# CONVERSION OF HYDROLYSATE TETANUS TOXIN TO TOXOID<sup>1</sup>

By EMANUEL B. SCHOENBACH, JULIUS J. JEZUKAWICZ, AND  
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(Received for publication November 12, 1942)

In the case of diphtheria toxin obtained by the growth of a suitable strain on a medium free from peptone, some slight difficulty has been experienced in bringing about complete detoxification with formalin (1). This is probably due to the relatively high content of both toxin and amino acid nitrogen present, and has necessitated the use of formalin up to 0.5 per cent concentration and several weeks' incubation. Under these conditions, successful conversion to toxoid occurs, and the remaining toxoid is an adequate antigen (2).

A number of small scale experiments have been carried out in order to learn how tetanus toxins, produced on similar media, will conduct themselves. These have been uniformly encouraging, and indicate that toxin of this sort should find application in human immunization. Unlike diphtheria toxin, a relatively low concentration of formalin is adequate, and an incubation period of about three weeks suffices.

The following experiment is presented: 400 cc. of the toxin produced in the experiment quoted elsewhere (3) and which had been filtered through a small Berkefeld "V" candle served for the experiment. Filtration was carried out March 18, 1941. The filtrate contained approximately 160,000 MLD per cc. for mice, and 50,000 for guinea pigs. Formalin in a quantity of 0.6 cc. (= 0.15 per cent) was added and the material incubated at 37° C. The treatment is tabulated below:

March 18	0.15 per cent formalin. Incubated 37° C.
March 28	Cold room 5.0 cc. in guinea pig—death in 5 days
April 2	37° C.
April 4	Cold room 5.0 cc. in guinea pig—death in 5 days
April 9	37° C.
April 14	Cold room 1.0 cc. in each of several guinea pigs—death in 10 to 14 days
April 25	37° C.

<sup>1</sup> Aided by a grant from the Commonwealth Fund.

April 26	0.05 per cent more formalin added
April 29	Cold room 5.0 cc. in each of 2 guinea pigs—both survived with no symptoms
May 5	1.0 cc. in each of 12 pigs
June 16	12 pigs all well. Each given 10 MLD of toxin (one of our own toxins identified as P-74 V). One unprotected control pig given 1.0 MLD of the same toxin died June 20 (101 hours).
June 26	Guinea pigs have shown no symptoms of tetanus during the 10-day interval. They were each bled a few cc. from the heart, the sera separated and pooled. Fifty per cent glycerol was added to the pooled sera and specimens sent to two manufacturers for an estimation of antitoxin content. The report <sup>2</sup> from one was "considerably more than $\frac{1}{10}$ unit but not quite $\frac{1}{2}$ unit per cc." The second reported "0.5 unit antitoxin per cc." Since the sample had been diluted with an equal volume of glycerol, the original pool must have contained approximately 1.0 unit antitoxin per cc.

A specimen of the toxoid was sent to the National Institute of Health, Bethesda, Maryland, for further appraisal of its antigenicity. They reported that "protection was afforded against 10 and 20 MLD's of toxin, 6 weeks after immunization with 1 cc."

It is clear that in this experiment the progress of detoxification was followed closely and the treatment accorded the toxin was directed by the toxicity to guinea pigs. As a result of several such experiments, it has become apparent that detoxification can be accomplished with considerable regularity in 3 weeks of incubation with 0.2 per cent formalin. The latter may be added all at one time, or in divided amounts, as in the experiment cited.

<sup>2</sup> We are indebted to Dr. A. L. Joyner and Miss Frances Clapp of the Lederle Laboratories, Inc., and to Dr. Bettylee Hampil of Sharp and Dohme, Inc., for these determinations.



## SUMMARY

Tetanus toxin, prepared on a peptone-free hydrolysate medium, can be detoxified with 0.2 per cent formalin in about 3 weeks' time. The resulting toxoid is sufficiently antigenic to meet the requirements of the National Institute of Health for a fluid tetanus toxoid designed for human use. The use of toxoid of this type for immunization should prevent any possibility of anaphylactic reactions due to antigenic components of certain peptones, heretofore widely used in tetanus toxin production.

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

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# A COMPARISON OF ANTIGENICITIES OF HYDROLYSATE AND PEPTONE TETANUS TOXOIDS IN THE GUINEA PIG<sup>1</sup>

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The resistance of the animal body to the injury caused by those bacterial agents which produce powerful exotoxins is commonly held to be the best understood of the various immune reactions. The toxins themselves, entirely free from the presence of the bacteria which produce them, are capable of eliciting all of the symptoms of the natural disease, and, as in the disease itself, death or recovery may ensue according to the quantity of toxin administered. Further, these toxins, being antigens by definition, will, under suitable conditions, stimulate the production in the body of anti-toxins capable of neutralizing specifically the ability of the same toxins to cause injury,—a neutralization which takes place in the test tube with almost mathematical precision and with the stoichiometric relationships of other chemical reactions. The toxins can be altered chemically, as for example by formalin, to a degree resulting in loss of ability to injure the body, while they still retain the original property of stimulating antibody production against the unaltered toxin. This last property provides the Public Health official and the physician with some of the most useful of tools for the prevention of disease, the practical value of which is now attested beyond a reasonable doubt.

It is a remarkable fact, however, that within this skeleton of definite information, there exists a mass of tradition in regard to certain details, accumulated over a period of many years, but founded either in uncontrolled experiment or in faulty observation. Many of these beliefs are held with a tenacity reminiscent of superstition, and are frequently responsible for actively retarding sound experimental advance.

Chief among these are the various dicta surrounding the laboratory preparation of toxins. For many years a perpetual source of difficulty to producers, certain statements in connection with

this process have been made so often, and with such great authority by so many workers that the absence of experimental support is no longer a source of concern. It would be simple to enumerate several of these rather fanciful beliefs, such as the one that potent toxin cannot be produced in summer,—probably explainable by the fact that hot weather is not conducive to human endeavor. The only one with which we propose to deal in this communication, however, is the old tradition that *toxin cannot be produced without peptone*, and the more recently drawn corollary, *even if it can be produced without peptone, it is no good*.

The former statement had become so ingrained in the minds of all bacteriologists, up to a few years ago, that the mere suggestion that diphtheria toxin, for example, might be obtained on a chemically defined medium was considered ridiculous. Peptone had always been found necessary; therefore, it was essential. Directing attention to the fact that growth of the organism had never been obtained without peptone resulted only in further statements that growth and toxin did not parallel each other (which is true) and that even if it were possible to grow the diphtheria bacillus on a peptone-free medium, no toxin would be obtained. Through the fortunate combination of Pappenheimer's (1) work on toxin, and experiments reported from this laboratory on growth (2), it was eventually possible to correct these erroneous impressions, and to demonstrate clearly that diphtheria toxin was a metabolic product of the growth of the bacteria under certain chemically controlled conditions (3).

Almost inevitably, then, the ability of the toxin, produced in this unorthodox manner, to behave like ordinary toxin was questioned. In particular, the competence of toxoid prepared from it to behave as an effective antigen was considered extremely doubtful. Even the fact that in toxicity and in antitoxin combining power it was considerably superior to the average material previously

<sup>1</sup> Aided by a grant from the Commonwealth Fund.

available, was held to be a disadvantage. Every manufacturer had seen toxin of an extremely low grade of potency produce antitoxin of astronomical proportions in horses,—or at least in *one* horse, whereas stronger toxins had often given poor results. Therefore, it was argued, since hydrolysate diphtheria toxin was relatively powerful, it would be a poor antigen. The general line of reasoning apparently questioned the fact that the toxin (or toxoid) was itself the antigen and suggested that the property of antigenicity resided primarily in some substance other than the specific poison.

Since there was no great urgency connected with the establishment of the inaccuracy of such a belief, as far as diphtheria toxoid was concerned, there seemed to be no point in carrying out elaborate experiments to establish the facts. The so-called "hydrolysate" toxoid has gradually come into production and use in certain localities, and there has been no evidence, so far as the writers are aware, of any lack of efficacy.

More important at the present time is the fact that the identical situation has now been transferred to the problem of tetanus toxoid. Employed on a tremendous scale for military immunization, its initial preparation on the traditional media containing Witte's peptone has had to be modified because of the presence, in that brand of peptone, at any rate, of a substance inducing severe anaphylaxis in certain individuals (4 to 6). Other varieties of peptone are still considered admissible, largely because no serious accidents have thus far been reported following their use. By their very nature as large fragments of protein, containing various enzyme residues and complex tissue components, their widespread use in man might well be avoided, provided the desired end may be reached without them.

These considerations have persuaded the writers to attempt the production on peptone-free media of tetanus toxin for conversion to toxoid for human use. A measure of success has attended these efforts (7 to 9), and it appears at this time to be worth while reporting a series of controlled experiments, designed to compare the antigenicity of hydrolysate toxoid with that of peptone toxoid. It seems to be a matter of some urgency that the facts in connection with this matter should be determined with accuracy and the

subject definitely removed from the realm of mere speculation.

It is unfortunate that there is not at this time a tetanus toxoid which has been established as a standard in the United States. Since, however, all tetanus toxoid on the market or supplied to the military forces has been checked tested at the Division of Biologics Control of the National Institute of Health and has been found to pass their minimum requirements, it would appear that specimens which have been distributed by a number of commercial producers to the trade or to the Army should constitute a reasonable standard with which a series of hydrolysate toxoids might be compared.

Proceeding on this assumption, we have obtained a number of lots of tetanus toxoid, representing products of several manufacturers, through the courtesy of the Office of the Surgeon General of the Army. The antigenicity of these toxoids

TABLE I

Guinea pig	Toxoid used	Serum titer		Results of toxin injection
		<i>Units anti-toxin per cc.</i>		
950	Commercial A	>1.0	<2.5	No symptoms
951	Commercial A	>2.5	<5.0	No symptoms
952	Commercial A	>0.25	<0.5	No symptoms
953	Commercial A	>0.25	<0.5	No symptoms
954	Commercial A	>2.5	<5.0	(Killed in bleeding)
955	Commercial A	>0.1	<0.25	Moderate symptoms, survived
914	Commercial B	>0.25	<0.5	Moderate symptoms, survived
915	Commercial B	>0.5	<1.0	No symptoms
916	Commercial B	>0.1	<0.25	Marked symptoms, survived
917	Commercial B	>0.01	<0.1	No symptoms
918	Commercial B	0.01		Moderate symptoms, survived
919	Commercial B	2.5		No symptoms
908	Commercial C	1.0		No symptoms
909	Commercial C	>0.1	<0.25	Moderate symptoms, survived
910	Commercial C	>1.0	<2.5	Moderate symptoms, survived
911	Commercial C	>1.0	<2.5	No symptoms
912	Commercial C	>0.1	<0.25	Slight symptoms, survived
913	Commercial C	>0.25	<0.5	No symptoms
920	Commercial D	0.25		Slight symptoms, survived
921	Commercial D	>0.1	<0.25	No symptoms
922	Commercial D	2.5		No symptoms
923	Commercial D	>2.5	<5.0	No symptoms
924	Commercial D	0.01		No symptoms
925	Commercial D	>1.0	<2.5	No symptoms
926	Commercial E	>2.5	<5.0	No symptoms
927	Commercial E	>0.25	<0.5	No symptoms
928	Commercial E	>0.25	<0.5	No symptoms
929	Commercial E	>0.01	<0.1	Moderate symptoms, survived
930	Commercial E	>0.25	<0.5	No symptoms
931	Commercial E	>0.1	<0.25	No symptoms
944	Commercial F	>0.001	<0.01	Severe symptoms, survived
945	Commercial F	>0.001	<0.01	Died, tetanus, second day
946	Commercial F	>0.001	<0.01	Died, tetanus, fourth day
947	Commercial F	0.01		Severe symptoms, survived
948	Commercial F	>0.25	<0.5	Slight symptoms, survived
949	Commercial F	>0.25	<0.5	No symptoms

TABLE II

Guinea pig	Toxoid used	Serum titer		Results of toxin injection
		<i>units anti-toxin per cc.</i>		
871	Hydrolysate Z	>0.1	<0.25	Moderate symptoms, survived
872	Hydrolysate Z	>0.001	<0.01	Moderate symptoms, survived
873	Hydrolysate Z	0.001		Died, tetanus, second day
874	Hydrolysate Z	>0.01	<0.1	No symptoms
875	Hydrolysate Z	>0.25	<0.5	No symptoms
876	Hydrolysate Z	0.01		Slight symptoms, survived
877	Hydrolysate AO	>0.25	<0.5	No symptoms
878	Hydrolysate AO	0.5		No symptoms
879	Hydrolysate AO	>0.25	<0.5	No symptoms
880	Hydrolysate AO	>0.1	<0.25	No symptoms
881	Hydrolysate AO	0.005		Died, tetanus, seventh day
882	Hydrolysate AO	>0.1	<0.25	No symptoms
885	Hydrolysate AQ	0.5		Slight symptoms, survived
886	Hydrolysate AQ	0.5		Slight symptoms, survived
887	Hydrolysate AQ	>0.25	<0.5	Slight symptoms, survived
888	Hydrolysate AQ	>0.001	<0.005	Moderate symptoms, survived
889	Hydrolysate AQ	0.5		No symptoms
890	Hydrolysate AQ	0.001		Died, tetanus, second day
890	Hydrolysate AT	>0.01	<0.1	Slight symptoms, survived
891	Hydrolysate AT	>0.25	<0.5	No symptoms
892	Hydrolysate AT	0.01		No symptoms
893	Hydrolysate AT	>0.25	<0.5	(Killed in bleeding)
894	Hydrolysate AT	0.005		No symptoms
895	Hydrolysate AT	0.25		No symptoms
788	Hydrolysate AV	0.25		Slight symptoms, survived
789	Hydrolysate AV	0.001		Died, tetanus, third day
790	Hydrolysate AV	>0.1	<0.25	Questionable symptoms, survived
791	Hydrolysate AV	>0.001	<0.01	Died, tetanus, second day
792	Hydrolysate AV	0.1		No symptoms
793	Hydrolysate AV	0.25		No symptoms
848	Hydrolysate BD	0.0025		Severe symptoms, recovered
850	Hydrolysate BD	>0.25	<0.5	No symptoms
851	Hydrolysate BD	0.1		Moderate symptoms, recovered
852	Hydrolysate BD	>5.0	<7.5	No symptoms
853	Hydrolysate BD	>0.1	<0.25	Moderate symptoms, survived
854	Hydrolysate BD	>0.25	<0.5	No symptoms

TABLE III

*Antitoxin titers of pools of sera from each lot*

Commercial A 0.8 units per cc.	Hydrolysate Z 0.1 units per cc.
Commercial B 0.4 units per cc.	Hydrolysate AO 0.3 units per cc.
Commercial C 0.5 units per cc.	Hydrolysate AQ 0.3 units per cc.
Commercial D 0.6 units per cc.	Hydrolysate AT 0.2 units per cc.
Commercial E 0.5 units per cc.	Hydrolysate AV 0.2 units per cc.
Commercial F 0.1 to 0.2 units per cc.	Hydrolysate BD 0.6 units per cc.

for guinea pigs and for man has been examined in parallel with a number of hydrolysate toxoids produced in this laboratory. The data in regard to guinea pig assay is presented herewith, while the results of human immunizations will be summarized in a separate communication.

#### METHOD

For a variety of reasons, the standard test method of the National Institute has not been followed. There is,

at present, no really suitable and generally accepted method of appraising antigenicity. We have chosen to adopt a procedure which should give the maximum information possible from the number of animals used.

A subcutaneous injection of 1.0 cc. of toxoid was given each of a series of guinea pigs, of approximately 250 grams per pig. Six or more animals were injected from each batch of toxoid to be examined. Included were toxoids from 6 different manufacturers, and 6 different lots of hydrolysate product. At the end of 6 weeks, the animals were bled from the heart, the individual sera separated and titrated for the antitoxin level. An occasional pig died as a result of the bleeding, but all others were injected, usually 48 hours later, with a massive (1000 MLD) dose of standard tetanus toxin (National Institute Lot H3), and the subsequent presence or absence of symptoms or time of death noted. The results are tabulated in Tables I, II, and III.

#### DISCUSSION

An inspection of the above data shows, in the first place, the tremendous individual variations among guinea pigs in ability to respond to identical stimuli. Tetanus toxoid is reputedly a relatively poor antigen when given initially, presumably because it represents a completely new experience for the tissue cells. Evidently, in certain animals, there is almost no antitoxin produced, while in others, the response is considerable. Assuming this to be an inherent vital property, it becomes problematical to what extent the differences indicated in the tables, between the commercial toxoids A and F, for example, demonstrate an actual dissimilarity in antigenicity of such magnitude.

If it were possible to obtain a genetically pure strain of guinea pigs with considerably greater uniformity of response, more significant comparative estimates would be possible. The only such strain now available, the so-called "Connaught," was employed in the test on hydrolysate AO quoted in the table, and in a repeat test on lot Z. The latter resulted in antitoxin titers of < 0.001, > 0.001 < 0.01, > 0.001 < 0.01, > 2.5 < 5.0, > 0.5 < 1.0, and > 2.5 < 5.0, with a pool of 0.5 units per cc. It is therefore apparent that these guinea pigs, also, differ in their ability to respond, and would be no more satisfactory than a mixed stock.

On the whole, as far as the results in guinea pigs go, it seems fairly obvious that there is no qualitative, and certainly very little, if any, quantitative difference in antigenic action between

available, was held to be a disadvantage. Every manufacturer had seen toxin of an extremely low grade of potency produce antitoxin of astronomical proportions in horses,—or at least in *one* horse, whereas stronger toxins had often given poor results. Therefore, it was argued, since hydrolysate diphtheria toxin was relatively powerful, it would be a poor antigen. The general line of reasoning apparently questioned the fact that the toxin (or toxoid) was itself the antigen and suggested that the property of antigenicity resided primarily in some substance other than the specific poison.

Since there was no great urgency connected with the establishment of the inaccuracy of such a belief, as far as diphtheria toxoid was concerned, there seemed to be no point in carrying out elaborate experiments to establish the facts. The so-called "hydrolysate" toxoid has gradually come into production and use in certain localities, and there has been no evidence, so far as the writers are aware, of any lack of efficacy.

More important at the present time is the fact that the identical situation has now been transferred to the problem of tetanus toxoid. Employed on a tremendous scale for military immunization, its initial preparation on the traditional media containing Witte's peptone has had to be modified because of the presence, in that brand of peptone, at any rate, of a substance inducing severe anaphylaxis in certain individuals (4 to 6). Other varieties of peptone are still considered admissible, largely because no serious accidents have thus far been reported following their use. By their very nature as large fragments of protein, containing various enzyme residues and complex tissue components, their widespread use in man might well be avoided, provided the desired end may be reached without them.

These considerations have persuaded the writers to attempt the production on peptone-free media of tetanus toxin for conversion to toxoid for human use. A measure of success has attended these efforts (7 to 9), and it appears at this time to be worth while reporting a series of controlled experiments, designed to compare the antigenicity of hydrolysate toxoid with that of peptone toxoid. It seems to be a matter of some urgency that the facts in connection with this matter should be determined with accuracy and the

subject definitely removed from the realm of mere speculation.

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952	Commercial A	>0.25	<0.5	No symptoms
953	Commercial A	>0.25	<0.5	No symptoms
954	Commercial A	>2.5	<5.0	(Killed in bleeding)
955	Commercial A	>0.1	<0.25	Moderate symptoms, survived
914	Commercial B	>0.25	<0.5	Moderate symptoms, survived
915	Commercial B	>0.5	<1.0	No symptoms
916	Commercial B	>0.1	<0.25	Marked symptoms, survived
917	Commercial B	>0.01	<0.1	No symptoms
918	Commercial B		0.01	Moderate symptoms, survived
919	Commercial B		2.5	No symptoms
908	Commercial C		1.0	No symptoms
909	Commercial C	>0.1	<0.25	Moderate symptoms, survived
910	Commercial C	>1.0	<2.5	Moderate symptoms, survived
911	Commercial C	>1.0	<2.5	No symptoms
912	Commercial C	>0.1	<0.25	Slight symptoms, survived
913	Commercial C	>0.25	<0.5	No symptoms
920	Commercial D		0.25	Slight symptoms, survived
921	Commercial D	>0.1	<0.25	No symptoms
922	Commercial D		2.5	No symptoms
923	Commercial D	>2.5	<5.0	No symptoms
924	Commercial D		0.01	No symptoms
925	Commercial D	>1.0	<2.5	No symptoms
926	Commercial E	>2.5	<5.0	No symptoms
927	Commercial E	>0.25	<0.5	No symptoms
928	Commercial E	>0.25	<0.5	No symptoms
929	Commercial E	>0.01	<0.1	Moderate symptoms, survived
930	Commercial E	>0.25	<0.5	No symptoms
931	Commercial E	>0.1	<0.25	No symptoms
944	Commercial F	>0.001	<0.01	Severe symptoms, survived
945	Commercial F	>0.001	<0.01	Died, tetanus, second day
946	Commercial F	>0.001	<0.01	Died, tetanus, fourth day
947	Commercial F		0.01	Severe symptoms, survived
948	Commercial F	>0.25	<0.5	Slight symptoms, survived
949	Commercial F	>0.25	<0.5	No symptoms

TABLE II

Guinea pig	Toxoid used	Serum titer		Results of toxin injection
		units anti-toxin per cc.		
871	Hydrolysate Z	>0.1	<0.25	Moderate symptoms, survived
872	Hydrolysate Z	>0.001	<0.01	Moderate symptoms, survived
873	Hydrolysate Z	0.001		Died, tetanus, second day
874	Hydrolysate Z	>0.01	<0.1	No symptoms
875	Hydrolysate Z	>0.25	<0.5	No symptoms
876	Hydrolysate Z	0.01		Slight symptoms, survived
877	Hydrolysate AO	>0.25	<0.5	No symptoms
878	Hydrolysate AO	0.5		No symptoms
879	Hydrolysate AO	>0.25	<0.5	No symptoms
880	Hydrolysate AO	>0.1	<0.25	No symptoms
881	Hydrolysate AO	0.005		Died, tetanus, seventh day
882	Hydrolysate AO	>0.1	<0.25	No symptoms
855	Hydrolysate AQ	0.5		Slight symptoms, survived
856	Hydrolysate AQ	0.5		Slight symptoms, survived
857	Hydrolysate AQ	>0.25	<0.5	Slight symptoms, survived
858	Hydrolysate AQ	>0.001	<0.005	Moderate symptoms, survived
859	Hydrolysate AQ	0.5		No symptoms
860	Hydrolysate AQ	0.001		Died, tetanus, second day
800	Hydrolysate AT	>0.01	<0.1	Slight symptoms, survived
801	Hydrolysate AT	>0.25	<0.5	No symptoms
802	Hydrolysate AT	0.01		No symptoms
803	Hydrolysate AT	>0.25	<0.5	(Killed in bleeding)
804	Hydrolysate AT	0.005		No symptoms
805	Hydrolysate AT	0.25		No symptoms
788	Hydrolysate AV	0.25		Slight symptoms, survived
789	Hydrolysate AV	0.001		Died, tetanus, third day
790	Hydrolysate AV	>0.1	<0.25	Questionable symptoms, survived
791	Hydrolysate AV	>0.001	<0.01	Died, tetanus, second day
792	Hydrolysate AV	0.1		No symptoms
793	Hydrolysate AV	0.25		No symptoms
848	Hydrolysate BD	0.0025		Severe symptoms, recovered
850	Hydrolysate BD	>0.25	<0.5	No symptoms
851	Hydrolysate BD	0.1		Moderate symptoms, recovered
852	Hydrolysate BD	>5.0	<7.5	No symptoms
853	Hydrolysate BD	>0.1	<0.25	Moderate symptoms, survived
854	Hydrolysate BD	>0.25	<0.5	No symptoms

TABLE III

*Antitoxin titers of pools of sera from each lot*

Commercial A 0.8 units per cc.	Hydrolysate Z 0.1 units per cc.
Commercial B 0.4 units per cc.	Hydrolysate AO 0.3 units per cc.
Commercial C 0.5 units per cc.	Hydrolysate AQ 0.3 units per cc.
Commercial D 0.6 units per cc.	Hydrolysate AT 0.2 units per cc.
Commercial E 0.5 units per cc.	Hydrolysate AV 0.2 units per cc.
Commercial F 0.1 to 0.2 units per cc.	Hydrolysate BD 0.6 units per cc.

for guinea pigs and for man has been examined in parallel with a number of hydrolysate toxoids produced in this laboratory. The data in regard to guinea pig assay is presented herewith, while the results of human immunizations will be summarized in a separate communication.

## METHOD

For a variety of reasons, the standard test method of the National Institute has not been followed. There is,

at present, no really suitable and generally accepted method of appraising antigenicity. We have chosen to adopt a procedure which should give the maximum information possible from the number of animals used.

A subcutaneous injection of 1.0 cc. of toxoid was given each of a series of guinea pigs, of approximately 250 grams per pig. Six or more animals were injected from each batch of toxoid to be examined. Included were toxoids from 6 different manufacturers, and 6 different lots of hydrolysate product. At the end of 6 weeks, the animals were bled from the heart, the individual sera separated and titrated for the antitoxin level. An occasional pig died as a result of the bleeding, but all others were injected, usually 48 hours later, with a massive (1000 MLD) dose of standard tetanus toxin (National Institute Lot H3), and the subsequent presence or absence of symptoms or time of death noted. The results are tabulated in Tables I, II, and III.

## DISCUSSION

An inspection of the above data shows, in the first place, the tremendous individual variations among guinea pigs in ability to respond to identical stimuli. Tetanus toxoid is reputedly a relatively poor antigen when given initially, presumably because it represents a completely new experience for the tissue cells. Evidently, in certain animals, there is almost no antitoxin produced, while in others, the response is considerable. Assuming this to be an inherent vital property, it becomes problematical to what extent the differences indicated in the tables, between the commercial toxoids A and F, for example, demonstrate an actual dissimilarity in antigenicity of such magnitude.

If it were possible to obtain a genetically pure strain of guinea pigs with considerably greater uniformity of response, more significant comparative estimates would be possible. The only such strain now available, the so-called "Connaught," was employed in the test on hydrolysate AO quoted in the table, and in a repeat test on lot Z. The latter resulted in antitoxin titers of  $< 0.001$ ,  $> 0.001 < 0.01$ ,  $> 0.001 < 0.01$ ,  $> 2.5 < 5.0$ ,  $> 0.5 < 1.0$ , and  $> 2.5 < 5.0$ , with a pool of 0.5 units per cc. It is therefore apparent that these guinea pigs, also, differ in their ability to respond, and would be no more satisfactory than a mixed stock.

On the whole, as far as the results in guinea pigs go, it seems fairly obvious that there is no qualitative, and certainly very little, if any, quantitative difference in antigenic action between

toxoids free from, and those containing, peptone. In other words, the theoretical aspect of the question, outlined in the introduction, appears to be born out. If the presence of "peptone," by which is meant relatively large, possibly non-diffusible fragments of protein, supplies and adjuvant effect on antigenicity, it must be a relatively feeble one.

The writers wish to thank Mr. William McBrearty for his technical assistance in carrying out this work.

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# ANTITOXIN RESPONSE IN MAN TO TETANUS TOXOIDS<sup>1</sup>

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The considerations leading to the experiments to be here described have been presented elsewhere (1). The final criterion by which one may judge the efficacy of a product designed to accomplish a particular purpose is its ability to fulfill this specific function. Consequently, it has appeared essential to determine whether tetanus toxoid, produced on a medium free from peptone, would compare favorably in actual human application with that prepared in the traditional manner on a peptone-containing medium. Fortunately, it is possible to determine the response directly with considerable accuracy by measuring the antitoxin produced in the individual's blood. To this extent, therefore, it is possible to appraise the relative merits of the two types of toxoid.

Actually, the final evidence of the ability of a presumably protected individual to survive, without symptoms, an injury which would normally lead to tetanus cannot be provided experimentally. Could such experiments be carried out, it is probable that the titrable level of antitoxin would prove to be the deciding factor. There is, naturally, a possibility that some type of tissue or local immunity might play a part, but there appears to be no experimental evidence to warrant such a supposition. Actually, the evidence from years of experience with passive protection overwhelmingly supports the view that circulating antitoxin is the important factor. Clinical tetanus can be prevented by maintaining an adequate level of heterologous antitoxin in the blood for a suitable length of time. A similar semi-permanent level of homologous antitoxin should be at least as effective. It seems reasonable, therefore, to judge the efficiency of tetanus toxoids on the basis of levels of antitoxin produced under similar conditions in man.

Tetanus toxoid for human immunization is employed in two forms, "fluid" and "alum precipitated." It is not our purpose to consider the

relative merits of the two at this time. Fluid toxoid presents the simplest set of conditions for study and the experiments to be described have been carried out with this type of material. In practical use, this substance is administered subcutaneously in 3 doses of 1.0 cc., at intervals of 3 to 4 weeks. This series of 3 primary vaccinations leads, in man, to a variable, but relatively weak, output of antitoxin, but does "condition" the tissues of an individual in such a way that a subsequent single injection of toxoid made after a lapse of months or years causes a rapid production of antibody. It is customary, therefore, to follow the primary series of 3 injections by a stimulating

TABLE I  
Effect of primary vaccination  
Units antitoxin per cc. serum

Commercial Toxoid A			
1.	>0.01		<0.1
2.	>0.25		<0.5
4.	>0.01		<0.1
5.	>0.25		<0.5
7.		0.5	
8.		0.25	
9.	>0.25		<0.5
10.	>0.001		<0.01
11.	>0.25		<0.5
12.	>0.25		<0.5
Commercial Toxoid B			
14.		0.01	
15.		0.1	
16.	>0.05		<0.1
17.	>0.001		<0.01 missed 3rd injection
18.	>0.25		<0.5
19.	>0.001		<0.01
20.	>0.01		<0.1
21.	>0.05		<0.1
22.	>0.001		<0.01
23.	>0.25		<0.5
24.	>0.001		<0.01
Commercial Toxoid C			
25.	>0.1		<0.25
26.	>0.1		<0.25
28.	>0.001		<0.01
29.		0.1	
30.	>0.1		<0.25
31.		0.001	
32.		2.5	
33.	>0.5		<1.0
34.		1.0	
36.	>0.001		<0.01

<sup>1</sup> Aided by a grant from the Commonwealth Fund.



TABLE I—Continued

*Hydrolysate Toxoid 186*

2.	>0.25	<0.5
6.	>0.25	<0.5
10.	>0.01	<0.1
11.	0.1	
12.	>0.005	<0.01
17.	>0.25	<0.5
20.	>0.25	<0.5
22.	>0.1	<0.25
27.	>0.1	<0.25
32.	0.1	
33.	>0.1	<0.25
34.	>0.001	<0.01
39.	>0.1	<0.25
42.	0.01	

*Hydrolysate Toxoid Z*

1.	0.1	
3.	>0.01	<0.1
5.	0.1	
7.	0.5	
9.	>0.25	<0.5
12.	>0.1	<0.25
15.	>1.0	<2.5
21.	0.1	
24.	0.5	
28.	>0.01	<0.1
29.	>0.1	<0.25
35.	>0.25	<0.5
38.	2.5	
40.	>0.25	<0.5

*Hydrolysate Toxoid AO*

4.	>0.05	<0.1
8.	>0.1	<0.25
16.	>1.0	<2.5
18.	0.01	
19.	>0.1	<0.25
23.	>0.1	<0.25
25.	>0.01	<0.1
26.	>0.1	<0.25
30.	0.25	
31.	>0.1	<0.25
36.	>0.25	<0.5
41.	>0.1	<0.25
43.	>0.1	<0.25
44.	>2.5	<5.0
45.	>0.1	<0.25

## TYPES OF TOXOIDS

As representative samples of commercial fluid toxoids, made on peptone-containing media, a number of vials produced by various manufacturers for use in the U. S. Army were obtained. All of these were from batches in actual use, which had certainly been check tested and found satisfactory. For comparison with these, several lots of "hydrolysate" (peptone-free) toxoid were selected. These had been prepared in our own laboratory by the

method described elsewhere (2) and had been packaged and tested for sterility and safety by the Massachusetts Antitoxin and Vaccine Laboratory.

## PLAN OF EXPERIMENTS

For the series of 3 primary vaccinations, groups of adult males, for the most part between 20 and 30 years of age, were selected. A group of between 10 and 15 individuals was used to test each toxoid. The men were given the customary 3 injections of 1.0 cc. each, at intervals of 3 to 4 weeks. Seven days after the third injection, 10 cc. of blood were collected from each man for titration of tetanus antitoxin.

To examine the response to the recall injection, similar groups were available of 12 men each who had received their primary tetanus inoculation with fluid toxoids about one year previously. The toxoids used at that time were all of the commercial peptone-containing type, and no record was available of the particular brands of toxoid used. These men were each bled 10 cc. for the determination of tetanus antitoxin still present as a result of their

TABLE II  
*Effect of stimulating dose*

Number	Pre-injection titer	Toxoid used	Post-injection titer
	units antitoxin per cc. serum		units antitoxin per cc. serum
1	>0.001	Commercial D	> 1.
2	0.001	Commercial D	> 5.
3	>0.01	Commercial D	> 1.
4	>0.01	Commercial D	2.5
5	>0.001	Commercial D	> 1.
6	>0.001	Commercial D	4.
7	>0.01	Commercial D	> 5.
8	>0.001	Commercial D	0.25
9	0.1	Commercial D	> 2.5
10	0.1	Commercial D	4.+
11	>0.01	Commercial D	> 4.
12	0.5	Commercial D	> 5.
13	>0.1	Hydrolysate 186	2.5
14	0.1	Hydrolysate 186	> 5.
15	>0.25	Hydrolysate 186	no blood
16	0.1	Hydrolysate 186	> 2.5
17	>0.01	Hydrolysate 186	> 5.
18	>0.1	Hydrolysate 186	> 4.
19	>0.01	Hydrolysate 186	2.5
20	>0.01	Hydrolysate 186	> 2.5
21	0.25+	Hydrolysate 186	> 5.
22	>0.01	Hydrolysate 186	> 2.5
23	>0.01	Hydrolysate 186	> 1.
24	>0.1	Hydrolysate 186	> 2.5
25	>0.01	Commercial E	> 5.
26	0.25	Commercial E	> 50.
27	2.5+	Commercial E	5.
28	>0.1	Commercial E	> 10.
29	>0.001	Commercial E	< 0.1
30	0.25	Commercial E	> 10.
31	>0.01	Commercial E	> 1.
32	>0.01	Commercial E	20.
33	>0.01	Commercial E	> 10.
34	0.1	Commercial E	> 10.
35	>0.25	Commercial E	> 10.
36	>0.01	Commercial E	5.
37	>0.1	Hydrolysate Lot Z	> 20.
38	>0.25	Hydrolysate Lot Z	> 1.
39	>0.01	Hydrolysate Lot Z	> 5.
40	0.5	Hydrolysate Lot Z	> 10.
41	0.1+	Hydrolysate Lot Z	> 1.
42	>0.25	Hydrolysate Lot Z	> 10.
43	0.01+	Hydrolysate Lot Z	> 4.
44	0.25	Hydrolysate Lot Z	> 10.
45	>0.1	Hydrolysate Lot Z	> 0.25
46	>0.1	Hydrolysate Lot Z	> 5.
47	0.01+	Hydrolysate Lot Z	> 0.25
48	>0.01	Hydrolysate Lot Z	> 1.

TABLE II—Continued

Number	Pre-injection titer	Toxoid used	Post-injection titer
	units antitoxin per cc. serum		units antitoxin per cc. serum
49	>0.1	Commercial F	5
50	0.25	Commercial F	> 5.
51	0.01+	Commercial F	5.
52	>0.01	Commercial F	> 1.
53	>0.01	Commercial F	< 2.5
54	0.1	Commercial F	> 1.
55	>0.001	Commercial F	5.
56	0.01+	Commercial F	< 2.5
57	0.01+	Commercial F	2.5+
58	0.5	Commercial F	> 1.
59	0.25	Commercial F	< 2.5
60	0.01+	Commercial F	> 5.
61	0.01+	Hydrolysate AO	> 30.
62	0.01+	Hydrolysate AO	> 1.
63	>0.01	Hydrolysate AO	< 2.5
64	>0.01	Hydrolysate AO	> 0.25
65	>0.01	Hydrolysate AO	< 0.5
66	0.1+	Hydrolysate AO	> 0.5
67	>1.	Hydrolysate AO	< 1.
68	>0.01	Hydrolysate AO	> 5.0
69	0.25	Hydrolysate AO	< 7.5
70	>0.01	Hydrolysate AO	> 5.0
71	>0.01	Hydrolysate AO	< 2.5
72	>0.01	Hydrolysate AO	> 1.
73	>0.01	Commercial G	< 2.5
74	>0.01	Commercial G	> 5.
75	0.01	Commercial G	< 10.
76	>0.01	Commercial G	> 2.5
77	>0.001	Commercial G	< 4.
78	>0.01	Commercial G	> 5.
79	>0.01	Commercial G	< 10.
80	>0.1	Commercial G	< 2.5
81	>0.01	Commercial G	> 10.
82	0.1+	Commercial G	< 20.
83	>0.1	Commercial G	> 1.
84	0.1+	Commercial G	< 2.5
85	0.01	Hydrolysate AT	> 10.
86	>0.1	Hydrolysate AT	< 20.
87	0.25	Hydrolysate AT	> 1.
88	>0.01	Hydrolysate AT	< 2.5
89	>0.001	Hydrolysate AT	> 2.5
90	0.001	Hydrolysate AT	< 5.
91	>0.001	Hydrolysate AT	> 5.
92	>0.1	Hydrolysate AT	< 10.
93	>0.25	Hydrolysate AT	> 5.
94	0.01+	Hydrolysate AT	< 5.
95	0.25+	Hydrolysate AT	> 20.
96	>0.01	Hydrolysate AT	< 30.
97	>0.01	Commercial H	> 2.5
98	>0.01	Commercial H	< 5.
99	0.25	Commercial H	> 5.
100	0.01+	Commercial H	< 10.
101	0.01+	Commercial H	> 5.
102	>0.25	Commercial H	< 2.5
103	>0.01	Commercial H	> 6.

earlier vaccination, and were then given, in groups of 12, 1 cc. of one of the toxoids. After a lapse of 7 days, blood was again collected for estimation of the response.

#### ANTITOXIN TITRATIONS

The serum was separated from the clots and the tetanus antitoxin in each sample was titrated in the usual manner, except that mice were employed rather than guinea pigs. The toxin used was a dry, standard preparation supplied by the National Institute of Health, and the quantities used at the various levels were those stated to be correct. A number of the sera were check-titrated at the National Institute by the guinea pig method, with perfectly satisfactory agreement between the two sets of results.

#### ANALYSIS OF RESULTS

It is evident from an inspection of these data that the response in antitoxin production obtained

with the various lots of hydrolysate toxoid compares favorably with that from those prepared on peptone media. The same rather considerable individual variation evidently occurs in man as in experimental animals, and a direct comparison of the various lots of toxoid used is difficult because of the relatively small size of the groups. It is perfectly evident, however, that neither as primary stimuli, nor when used as recall injections, do the hydrolysate toxoids differ in any degree from the peptone products.

The following statistical analysis of the figures has been prepared by Miss Jane Worcester of the Department of Vital Statistics, Harvard School of Public Health.

"Since antitoxin titers are necessarily positive, it might seem better to discuss their logarithms rather than the actual values, but a cursory examination of the data indicates that because of the nature of the variation neither the logarithms nor the values themselves form a satisfactory series upon which to use precise statistical techniques. Consequently a rather general discussion seems preferable to one aiming at precision. The tables give the mean and median response to the commercial and hydrolysate toxoids.

#### Bleedings seven days after third injection of 1.0 cc.

	Mean	Median		Mean	Median
Commercial A	0.28	0.38	Hydrolysate 186	0.18	0.18
Commercial B	0.10	0.06	Hydrolysate Z	0.52	0.28
Commercial C	0.49	0.18	Hydrolysate AO	0.51	0.18
Total	0.28	0.10	Total	0.41	0.18

The means and medians indicate that the effects of commercial toxoids A and C cannot be considered different, but that it is possible that the effect of B is less than the other two. There is no difference between the members of the hydrolysate series. If the data are combined into two series, one for the commercial and one for the hydrolysate preparations, the mean values are 0.28 and 0.41. The difference between the medians (0.10 and 0.18) is slightly smaller than that between the means and seems to be too small to have any significance.

The post-recall titers present much the same picture.

#### Post-recall titers

	Mean	Median		Mean	Median
Commercial D	3.9	3.9	Hydrolysate 186	4.2	3.8
Commercial B	14.1	15.0	Hydrolysate Z	8.0	6.0
Commercial E	6.5	4.4	Hydrolysate AO	4.5	3.5
Commercial F	6.6	7.5	Hydrolysate AT	4.3	3.8
Commercial C	5.4	6.0	Hydrolysate		
Total	7.5	5.0	Total	5.3	3.8

With the exception of the effect produced by the toxoid B, the various products seem to be acting in the same way. The mean values for the total groups are 7.5 and 5.3. If the values for B are removed, the mean value for the commercial series becomes 5.6 which does not differ from 5.3. This particular lot of toxoid B does seem to be producing a significantly better titer than any of the other toxoids considered."

It is not without interest that toxoid B, used in the primary vaccination series, and toxoid B used in the stimulation experiment, were identical, the same lot from the same manufacturer. In the first case, it apparently was the least effective of any, whereas in the second group it appeared to be outstandingly good. The same toxoid showed a perfectly average result when tested in guinea pigs (1).

The use of peptone-free tetanus toxoid for human prophylaxis thus appears completely satisfactory from the standpoint of antitoxin production. It is neither more nor less effective than the ordinary type, but unlike that, it cannot lead, when used initially, to sensitization to some component of the original medium. Naturally it contains protein products of the tetanus bacillus, including toxoid, and in certain individuals, reactions due to these materials may occur, either on the first or a subsequent injection. It is generally believed that such reactions are few in number and mild,—quite different from the immediate and alarming anaphylactic type which has occurred in certain individuals, and has been shown to be due to components of peptone (3). For example, in the series of approximately 50 primary human vaccinations, no reactions were noted, although the majority of the volunteers in these groups were men with definite histories of al-

lergy. In one man who had received two courses of (probably) alum-precipitated toxoid over a period of several years, a stimulating dose of one of the hydrolysate toxoids resulted in a badly swollen arm, but no general symptoms. His serum antitoxin increased from 2.5 to 12 units per cc. in 7 days. Unfortunately, it was impracticable to keep the remainder of the men under close observation, but no alarming symptoms were reported in any case.

It now seems possible, therefore, to prepare this prophylactic agent in such form that the only antigenic material present is the one desired, plus such additional products of growth of the tetanus bacillus as naturally accompany it. Whether an effort to purify further the toxoid itself, by removal of these latter substances, will be desirable must depend on more extended practical use of the material.

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

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# BLOOD AMYLASE ACTIVITY IN DISEASE OF CARBOHYDRATE METABOLISM AND IN NON-DIABETIC PANCREATIC DISEASE<sup>1</sup>

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Thus far, no role has been established for the enzyme amylase in the intermediate carbohydrate metabolism. The recent work of Cori and co-workers (1, 2), and of Ostern, Herbert, and Holmes (3), makes it appear highly improbable that amylase participates in the carbohydrate metabolism of liver, muscle, and other tissues. A previous communication (4) reported enzyme studies on the liver tissue in glycogen-storage disease (von Gierke's disease), which failed to demonstrate any definite abnormality in liver amylase activity in this disease of carbohydrate metabolism. Nevertheless, abnormal blood amylase activity has often been reported in the common diseases of carbohydrate metabolism—diabetes and liver disease. In fact, only a single report (Nørby (5)) recording no alteration in the enzyme activity in diabetes is encountered. However, among the numerous other reports, there is marked divergence of opinion concerning the nature of the abnormality in amylase activity. Almost as many papers are found favoring an increase in blood amylolytic activity in these conditions as there are reporting the opposite, and indeed, experimental work is often presented to corroborate the findings, whether they be high or low (6).

These circumstances prompted a reinvestigation of blood amylase activity, with a revised method, in patients suffering from diabetes, hepato-cellular and other kinds of liver disease, and in a patient with glycogen-storage disease. In order to obtain a basis for comparison, similar determinations were carried out in a series of normal subjects and also in several children suffering from various

childhood diseases which do not affect the carbohydrate metabolism.

In sharp contrast with the divergent reports on blood amylase activity in disturbances of carbohydrate metabolism is the marked agreement of results recorded in acute disease involving the excretory function of the pancreas. Schlesinger (7), in 1908, demonstrated that ligation of the pancreatic ducts in animals produced increased blood amylase activity. Since that time, augmented blood amylase activity has been shown, by numerous authors, to bear a definite clinical and experimental relationship to this type of pancreatic disease, as is pointed out in recent articles (8, 9). It was, therefore, considered advisable also to determine the blood amylase activity in this disease category with the revised method.

The basis for an entirely reliable method for the determination of blood amylase activity lies in the following facts: (1) The hydrolysis of starch to reducing sugars by amylase takes place as a monomolecular reaction during a certain phase of the splitting (10, 11); and (2) these sugars, chiefly maltose, can be measured with a high degree of accuracy. A method was devised which includes fractional splitting as an integral part of the procedure, thereby assuring measurement of the hydrolysis during its monomolecular phase. Hanes' (12) modification of the Hagedorn-Jensen method was employed to estimate the reducing sugars formed, since this method yields accurate results for such sugars in the presence of starch. The amylase method, therefore, is based on the saccharifying power of the enzyme. This basis of measuring amylolytic activity was first popularized by Kjeldahl<sup>3</sup> in 1880. Since that time the saccharification method has been employed in modified form by many authors including Sherman *et al.* (10), Willstätter *et al.* (11), Somogyi

<sup>1</sup> Work aided by a grant from the Rockefeller Foundation, the Bingham Associates Fund, and the Charlton Fund.

<sup>2</sup> Work completed during tenure of Emanuel Libman Fellowship.

<sup>3</sup> Quoted by Sherman *et al.* (10).

With the exception of the effect produced by the toxoid B, the various products seem to be acting in the same way. The mean values for the total groups are 7.5 and 5.3. If the values for B are removed, the mean value for the commercial series becomes 5.6 which does not differ from 5.3. This particular lot of toxoid B does seem to be producing a significantly better titer than any of the other toxoids considered."

It is not without interest that toxoid B, used in the primary vaccination series, and toxoid B used in the stimulation experiment, were identical, the same lot from the same manufacturer. In the first case, it apparently was the least effective of any, whereas in the second group it appeared to be outstandingly good. The same toxoid showed a perfectly average result when tested in guinea pigs (1).

The use of peptone-free tetanus toxoid for human prophylaxis thus appears completely satisfactory from the standpoint of antitoxin production. It is neither more nor less effective than the ordinary type, but unlike that, it cannot lead, when used initially, to sensitization to some component of the original medium. Naturally it contains protein products of the tetanus bacillus, including toxoid, and in certain individuals, reactions due to these materials may occur, either on the first or a subsequent injection. It is generally believed that such reactions are few in number and mild,—quite different from the immediate and alarming anaphylactic type which has occurred in certain individuals, and has been shown to be due to components of peptone (3). For example, in the series of approximately 50 primary human vaccinations, no reactions were noted, although the majority of the volunteers in these groups were men with definite histories of al-

lergy. In one man who had received two courses of (probably) alum-precipitated toxoid over a period of several years, a stimulating dose of one of the hydrolysate toxoids resulted in a badly swollen arm, but no general symptoms. His serum antitoxin increased from 2.5 to 12 units per cc. in 7 days. Unfortunately, it was impracticable to keep the remainder of the men under close observation, but no alarming symptoms were reported in any case.

It now seems possible, therefore, to prepare this prophylactic agent in such form that the only antigenic material present is the one desired, plus such additional products of growth of the tetanus bacillus as naturally accompany it. Whether an effort to purify further the toxoid itself, by removal of these latter substances, will be desirable must depend on more extended practical use of the material.

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

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# BLOOD AMYLASE ACTIVITY IN DISEASE OF CARBOHYDRATE METABOLISM AND IN NON-DIABETIC PANCREATIC DISEASE<sup>1</sup>

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(Received for publication July 2, 1942)

Thus far, no role has been established for the enzyme amylase in the intermediate carbohydrate metabolism. The recent work of Cori and co-workers (1, 2), and of Ostern, Herbert, and Holmes (3), makes it appear highly improbable that amylase participates in the carbohydrate metabolism of liver, muscle, and other tissues. A previous communication (4) reported enzyme studies on the liver tissue in glycogen-storage disease (von Gierke's disease), which failed to demonstrate any definite abnormality in liver amylase activity in this disease of carbohydrate metabolism. Nevertheless, abnormal blood amylase activity has often been reported in the common diseases of carbohydrate metabolism—diabetes and liver disease. In fact, only a single report (Nørby (5)) recording no alteration in the enzyme activity in diabetes is encountered. However, among the numerous other reports, there is marked divergence of opinion concerning the nature of the abnormality in amylase activity. Almost as many papers are found favoring an increase in blood amylolytic activity in these conditions as there are reporting the opposite, and indeed, experimental work is often presented to corroborate the findings, whether they be high or low (6).

These circumstances prompted a reinvestigation of blood amylase activity, with a revised method, in patients suffering from diabetes, hepato-cellular and other kinds of liver disease, and in a patient with glycogen-storage disease. In order to obtain a basis for comparison, similar determinations were carried out in a series of normal subjects and also in several children suffering from various

childhood diseases which do not affect the carbohydrate metabolism.

In sharp contrast with the divergent reports on blood amylase activity in disturbances of carbohydrate metabolism is the marked agreement of results recorded in acute disease involving the excretory function of the pancreas. Schlesinger (7), in 1908, demonstrated that ligation of the pancreatic ducts in animals produced increased blood amylase activity. Since that time, augmented blood amylase activity has been shown, by numerous authors, to bear a definite clinical and experimental relationship to this type of pancreatic disease, as is pointed out in recent articles (8, 9). It was, therefore, considered advisable also to determine the blood amylase activity in this disease category with the revised method.

The basis for an entirely reliable method for the determination of blood amylase activity lies in the following facts: (1) The hydrolysis of starch to reducing sugars by amylase takes place as a monomolecular reaction during a certain phase of the splitting (10, 11); and (2) these sugars, chiefly maltose, can be measured with a high degree of accuracy. A method was devised which includes fractional splitting as an integral part of the procedure, thereby assuring measurement of the hydrolysis during its monomolecular phase. Hanes' (12) modification of the Hagedorn-Jensen method was employed to estimate the reducing sugars formed, since this method yields accurate results for such sugars in the presence of starch. The amylase method, therefore, is based on the saccharifying power of the enzyme. This basis of measuring amylolytic activity was first popularized by Kjeldahl<sup>3</sup> in 1880. Since that time the saccharification method has been employed in modified form by many authors including Sherman *et al.* (10), Willstätter *et al.* (11), Somogyi

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(13), and others. The method described in this paper offers the advantage derived from the fractional splitting which is incorporated. This makes it possible to ascertain in each instance whether or not the determination was made during the monomolecular phase of amylolysis.

METHOD

There are three parts: I. The enzymatic hydrolysis of starch; II. deproteinization of serum proteins; III. determination of the reducing substances formed. Interruption may be made between any of the parts.

The reagents used are as follows: Substrate, 1.4 per cent water clear solution of soluble starch (Kalbaum-Zulkowsky); M/15 Na<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> buffer having a pH of 6.8; activator, M/5 NaCl; 1N HCl and NaOH; 10 per cent neutral sodium tungstate<sup>4</sup>; and the reagents for the Hanes' method.

*Part I.* Place 25 cc. of substrate, 15 cc. of buffer, 5 cc. of activator, and 2.5 cc. of distilled water in a 125 cc. Erlenmyer flask. The latter is immersed in a thermostatically controlled waterbath, kept at 37° C. for the duration of the hydrolysis. When the temperature of the contents of the flask reaches 37° C., 2.5 cc. of serum are added with continuous shaking, and immediately thereafter 10 cc. of the flask's contents are transferred to a 15 cc. centrifuge tube containing 1 cc. of 1N HCl (the HCl inactivates the enzyme and also facilitates the precipitation of the proteins in Part II). The time is noted. Three subsequent 10 cc. samples are similarly treated at exactly 20, 40, and 60 minutes later. Thus, 4 aliquot fractions are obtained, each containing 0.5 cc. of serum. The first fraction removed serves as the blank.

*Part II.* Deproteinization is accomplished by adding 2 cc. of the sodium tungstate to each fraction, stirring, and centrifuging for 5 minutes. The water clear supernatant fluid is decanted into 50 cc. Hagedorn-Jensen tubes, each of which contains 1 cc. of 1N NaOH to neutralize the HCl used in Part I.

*Part III.* The amount of reducing substance in each fraction is measured by the Hanes' method.

The method of calculating the amylolytic activity and several examples to illustrate the findings in normal human serum are given in Tables I and II. The average 20-minute sodium thiosulfate titration value is multiplied by 2, to obtain the value for 1 cc. of serum, and by the conversion factor for maltose (0.55). The product thus obtained is multiplied by 3 to obtain the amount split in 1 hour (*i.e.*, the measure of the amylolytic activity of the serum).

It will be seen from Tables I and II that a straight line type of curve is obtained if the amount of reducing substance found in the fractions is plotted against the time elapsed.

<sup>4</sup> Since the samples must be neutral before Part III is carried out, the 1N HCl and the NaOH must be made up accurately, and the sodium tungstate should give a neutral reaction with litmus.

TABLE I  
*Normal human serum*

Subject	N/75 Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> titration values of the fractions			
	Blank	20 minutes	40 minutes	60 minutes
A	7.07	6.07	5.08	4.0
B	6.80	5.00	3.30	1.4
C	6.96	5.86	4.78	3.6
D	7.90	7.08	6.28	5.4
E	7.19	6.80	6.44	6.0

TABLE II  
*Calculation of amylolytic activity*

*Titration value of blank	7.07	7.07
Titration value of fractions	6.07	5.08
Increment in 20, 40, 60 minutes	1.00	1.99
Increment in each 20 minutes	1.00	0.995
Average 20-minute titration value = 1.00 cc.		
$1.00 \times 2 \times 0.890\dagger \times 0.55 \times 3$		
= 2.94 mgm. maltose 1 cc. s		
1 hour		

\* The titration values are those of Subject A in Table I.  
† Correction factor for N/75 sodium thiosulfate used.

*Modification of method for rapid determination.* accurate rapid determination in serum of high amylolytic activity may be carried out by making the following modifications in the method. Add 0.5 cc. of serum and 4.5 cc. of water, instead of the quantities indicated above, to the flask. Limit the hydrolysis to two 10-minute periods. With the modified method, the amylase value in serum of high activity can be ascertained in less than one hour.

NORMAL SUBJECTS

The individuals examined included patients and healthy persons who had no evidence of organic disease, physicians and other hospital workers. It will be seen that the amylolytic values, as shown in Table III, range between

TABLE III  
*Serum amylolytic activity in normal subjects*

Mgm. maltose split in 1 hour	Number of subjects
0.5 to 1	1
1 to 2	5
2 to 3	11
3 to 4	4
4 to 5	3
5 to 5.5	1
Total subjects	25
Average splitting	2.6 mgm.

and 5.5 mgm. (average 2.6 mgm.) of maltose split by 1 cc. of serum in 1 hour. For this reason a unit of amylolytic activity for clinical purposes was established as equal to 1 mgm. of maltose formed by 1 cc. of serum in 1 hour under the conditions utilized in the method.

## DIABETIC PATIENTS

The blood of 56 diabetic patients was tested for its amylase activity. The majority of the patients were ambulatory. All grades of diabetes, with the exception of diabetic coma, and various age groups, were included. The results disclose the same range of amylolytic activity as was found in the normal individuals. No relationship to insulin therapy could be established.

TABLE IV

*Serum amylolytic activity in diabetic patients*

Amylase units	Number of patients
0.5 to 1	1
1 to 2	15
2 to 3	18
3 to 4	11
4 to 5	9
5 to 6	2
Total number of patients	56
Average activity in units	2.7

## PATIENTS WITH LIVER DISEASE

This series includes 5 cases of toxic hepatitis, 1 of acute yellow atrophy, 5 of Laennec's cirrhosis (1 with intercurrent jaundice), 3 of cholelithiasis presenting no symptoms of pancreatic involvement, and 4 cases of obstructive icterus. The latter were of varied etiology: common duct stone, xanthomatous biliary cirrhosis, and carcinoma of the head of the pancreas. One case of congenital hemolytic icterus and one of hepatomegaly in a child with essential lipemia are also included in this group. From the findings shown in Table

TABLE V

*Serum amylolytic activity in liver disease*

Disease	Amylase units
Acute yellow atrophy	2.70
Toxic hepatitis	2.25
Toxic hepatitis	3.10
Toxic hepatitis	1.50
Toxic hepatitis	4.60
Toxic hepatitis	5.00
Cirrhosis	1.80
Cirrhosis	4.00
Cirrhosis	2.90
Cirrhosis	1.60
Cirrhosis with intercurrent hepatitis	2.60
Cholelithiasis	1.10
Cholelithiasis	5.25
Cholelithiasis	2.05
Hepatomegaly (Essential lipemia)	2.33
Hemolytic icterus (Congenital)	1.30
Obstructive icterus (Cholelithiasis)	0.85
Obstructive icterus (Cholelithiasis)	2.25
Obstructive icterus (Xanthomatous biliary cirrhosis)	3.30
Obstructive icterus (Carcinoma of head of pancreas)	43.00

V, it is seen that values similar to those in normal individuals were obtained in all instances except the one in which there was pancreatic involvement due to carcinoma of the head of the pancreas.

## PATIENT WITH GLYCOGEN STORAGE DISEASE

A value of 2.2 units was found in the blood of a 3-day-old infant suffering from this disorder of carbohydrate metabolism.<sup>5</sup> Autopsy subsequently revealed typical findings of markedly increased glycogen storage in the liver.

## PATIENTS WITH VARIOUS CHILDHOOD DISEASES

The results of blood amylase determinations in 7 children suffering from a variety of diseases are given in Table VI. The values fell within the range found in the normal subjects.

TABLE VI

*Serum amylolytic activity in disease in childhood*

Chorea	5.50
Pneumonia	2.90
Rheumatic fever	2.25
Rheumatic carditis	3.10
Meckel's diverticulum	2.30
Essential xanthomatosis	2.40
Nephritis	4.35

## PATIENTS WITH NON-DIABETIC PANCREATIC DISEASE

Frequent determinations were made in 4 cases of disease involving the excretory function of the pancreas (Table VII). One of the cases is that

TABLE VII

*Serum amylase in non-diabetic pancreatic disease*

Patient A		Patient B		Patient C		Patient D	
Carcinoma of head of pancreas		Acute pancreatic edema		Acute pancreatic edema		Acute pancreatic edema	
Date	Amylase units	Date	Amylase units	Date	Amylase units	Date	Amylase units
April 27	39.95	May 4	24.04	May 17	55.29	May 25	49.20
May 2	43.71	May 5	12.70	May 18	9.07	May 27	17.14
May 4	43.02	May 7	3.25	May 19	8.10	May 23	8.23
May 7	35.15	May 12	3.00	May 21	7.20	May 20	1.60
May 25	40.60			May 22	5.40		
May 30	50.40			May 23	5.90		
				May 24	5.40		

of the patient with carcinoma of the head of the pancreas included in Table VI. The other 3 were those of patients who entered the hospital with a

<sup>5</sup> The blood from this patient was obtained through the kindness of Dr. Elmer Barron and Dr. Francis MacDonald of the Boston Floating Hospital.



clinical picture characteristic of acute pancreatitis.<sup>6</sup> The subsequent course and the clinical investigations carried out made it possible to establish the diagnosis of acute pancreatic edema, secondary to cholelithiasis, in each of the cases.

A maintained increase of the blood amylase was present in the patient with the carcinoma of the head of the pancreas. The patients suffering from acute pancreatic edema exhibited initial elevations of considerable magnitude with a subsequent return of the amylase activity to a normal level within several days.

#### DISCUSSION

*Non-diabetic pancreatic disease.* The increased blood amylase activity as observed in all of the 4 cases in this group is in accord with the long established relationship between high blood amylase values and disease of this type. The short duration of the increased activity in acute pancreatitides has also been well established clinically (14, 15) and experimentally (16), and the clinical value of the blood amylase determination in these conditions has been stressed (17). A maintained increase of amylase activity as observed in the patient with carcinoma of the head of the pancreas has not been reported previously. However, a persistent increase in serum amylase activity has been observed in dogs following permanent experimental closure of the pancreatic ducts (18).

*Diseases of carbohydrate metabolism.* The patients with diabetes, hepato-cellular liver disorders, and the one patient with glycogen storage disease, all exhibited normal blood amylase values. These findings are in keeping with the fact that no role has been found in the intermediary carbohydrate metabolism for this enzyme, despite the recent advancement of knowledge in this field. Recent contributions (2, 3) demonstrate the presence in liver, muscle, and other tissues, of a reversible glycogen-glucose conversion mechanism which depends on a specific phosphorylase-phosphatase enzyme system. This work also supplies experimental evidence that the mechanism is capable by itself of fulfilling the body's requirements for glucose obtained from glycogen stored in the liver,

and makes it appear highly improbable that amylase participates in this essential phase of intermediary carbohydrate metabolism (1).

Liver tissue possesses amylolytic activity, but this activity probably results mainly from the amylase contained in the hepatic blood and not in the liver cells proper (19, 1, 8). However, regardless of its source, there is no proof that the amylase present in the liver has a function in carbohydrate metabolism. Conversely, there are reasons which make it doubtful that it has any function in the liver. The activity of the enzyme which is but slight in human blood is even less marked in liver tissue. In duodenal juice, where it plays a known role in the digestion of carbohydrates, the amylase is more than one thousand times<sup>7</sup> as active as that of blood or liver. Furthermore, amylase hydrolyzes starches to dextrins and to maltose as an end product (20), and, in physiological concentration, not at all or only in small amounts to glucose (21). The presence of the chief split products of amylolysis, maltose and non-fermentable reducing substances, cannot be demonstrated in liver tissue (8). Were maltose to be formed as an intermediary product and rapidly converted to the tissue utilizable glucose, the aid of a highly active maltase would be required. The latter enzyme is not known to be present in the human liver.

At present, therefore, there is no proof of the participation of amylase in the intermediary carbohydrate metabolism. The recent work and the other evidence mentioned militate against such an assumption. In accord with this are the normal blood amylase findings in diseases of carbohydrate metabolism, as reported in this paper. These findings may, therefore, be considered as clinical evidence pointing in the same direction.

#### SUMMARY AND CONCLUSIONS

1. The blood amylolytic activity of 25 normal patients was found to be between 0.5 and 5.5 mgm. (average 2.6 mgm.) of maltose per cc. of serum per hour, with the method described.

2. A clinical unit of amylolytic activity was established as equal to 1 mgm. of maltose formed in 1 hour by 1 cc. of serum.

<sup>6</sup> The study of these patients was made possible through the cooperation of Dr. Stephen Maddock, Director of the Surgical Research Laboratories of the Boston City Hospital.

<sup>7</sup> Unpublished data.

3. Normal blood amylase values were obtained in 56 diabetics, 11 patients with hepato-cellular liver disease, 8 with other types of liver disturbance, and 1 with glycogen storage disease.

4. Markedly increased blood amylase activity of transitory duration was observed in 3 patients with acute pancreatic edema; similarly augmented activity, but of a persistent nature, was found in a patient suffering from a carcinoma of the head of the pancreas.

\* 5. The increased blood amylase activity encountered in the 4 patients with disease involving the excretory function of the pancreas is in accord with the clinical and experimental findings of numerous other authors.

6. The normal blood amylase values in disease of carbohydrate metabolism is offered as clinical evidence of the non-participation of amylase in the intermediary carbohydrate metabolism.

The author gratefully acknowledges the guidance given him by Dr. S. J. Thannhauser in carrying out this study.

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# NITROGEN RETENTION, CREATINURIA, AND OTHER EFFECTS OF THE TREATMENT OF SIMMONDS' DISEASE WITH METHYL TESTOSTERONE

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One of the immediate effects of hypophysectomy is a loss of nitrogen (1) and a corresponding loss in weight (2). This change is reflected clinically by the so-called cachexia of Simmonds' disease (3). Nitrogen balance is then re-established at a lower level of body weight with lessened food intake.

In the experimental animal, adequate replacement therapy is readily obtained with anterior hypophyseal substance or extracts (4, 5), including the production of nitrogen retention and gain in weight (1). Clinically, however, such attempts have been singularly unsuccessful (6, 35) except for one case treated with pregnant mare's serum (7), and another recent report by Williams (8), of treatment with a mixture of thyroid, desoxycorticosterone, and methyl testosterone.

The recent reports of growth in cases of dwarfism treated by methyl testosterone (9) reveal that nitrogen retention and creatinuria are produced. The origin of some types of dwarfism has been attributed by many to a disturbance in the growth function of the anterior hypophysis. This suggests that methyl testosterone may produce its effects on nitrogen metabolism in the absence of hypophyseal function, which is of great interest in view of the intimate role of the hypophysis in such metabolism (10). However, since the cause of the dwarfism cannot be definitely localized to the hypophysis (11), it would seem important to try methyl testosterone in cases of Simmonds' disease, in which hypophyseal function is known to be lacking and in which any effect on nitrogen metabolism is not complicated by concurrent growth. Positive results might well point to this hormone as a possible therapeutic agent in this disease.

Accordingly, a study was conducted in the

Presbyterian Hospital on two cases of Simmonds' disease due to destruction of the hypophysis by intrasellar tumors, a girl of 20 years and a man of 31. They were placed on a low creatine diet and balance studies were conducted before, during, and after treatment with methyl testosterone. Striking subjective, objective, and laboratory changes occurred. Two additional cases were treated and followed clinically, with the production of similar changes, although it was not possible to secure adequate laboratory data. These are mentioned briefly at the end of the text and their case histories are appended.

## METHODS

The case histories of the 2 patients used in this study are detailed at the end of this paper. Both were placed on low creatine diets, the man receiving 2,450 calories per day composed of 290 grams of carbohydrate, 75 grams of protein, and 110 grams of fats; the girl received 1,600 calories divided into 200 C, 55 P, and 65 F, calculated from diet tables. The man carried on his customary activities; the girl was unable to do so, due to weakness, and remained in bed. All urine was pooled as 24-hour outputs under toluol, for analysis of total nitrogen, creatine, creatinine, uric acid, and phosphorus. Occasional 24-hour specimens were collected with chloroform for 17-ketosteroid determination. Nitrogen was determined by a modified micro-Kjeldahl method (12), creatine and creatinine by a modification of the Folin method (13), uric acid by the Brown method (14), and phosphorus by the Fiske and Subbarow method (15). Urinary 17-ketosteroids were determined colorimetrically by the Zimmermann procedure as detailed by Callow and Callow (16), following an extraction procedure previously outlined (17). Bloods for cholesterol, sugar, and serum protein partition were determined by the Bloor (18), Folin and Wu (19), and Howe (20), methods respectively. Basal metabolic rates were run in a closed system apparatus, using the DuBois formula for surface area calculations. Crystalline methyl testosterone,<sup>2</sup> 10 mgm. tablets each,

<sup>2</sup>Dr. Edward Henderson of the Schering Corporation generously supplied the methyl testosterone and the placebo tablets. Mr. Mautner of the Ciba Corporation furnished a small quantity of methyl testosterone, also.

<sup>1</sup>Aided by a grant from the Rockefeller Foundation for Teaching and Research in Neurology.

TABLE I  
Table listing the urinary excretion values for nitrogen, creatine, creatinine, inorganic phosphorus, and uric acid in Simmonds' disease, before, during, and after methyl testosterone administration  
Patient R. K., female, on a diet containing 8.8 grams of protein N per day, less 0.4 grams N in stool

Day of experiment. Start—April 21, 1942	Treatment	Nitrogen	Creatinine	Creatine	Phosphorus	Uric acid
		grams per 24 hours		mgm. per 24 hours		
1 to 7 8 to 15 16 to 22	Methyl testosterone	3.35 3.60 3.19	0.617 0.600 0.560	0.369 0.433 0.354	Average 0.460	Average 0.216
23 to 30 31 to 38 39 to 46 47 to 54 55 to 62	None	5.12 6.11 6.82 6.71 6.53	0.765 0.706 0.744 0.696 0.708	0.493 0.343 0.317 0.244 0.196	Average 0.620	Average 0.306
63 to 70 71 to 77	Methyl testosterone	4.47 4.26	0.693 0.700	0.202 0.357	Average 0.490	Average 0.282

Patient McA., male, on a diet containing 12.0 grams protein N per day, less 1.1 grams N in stool

Start—May 1, 1942 1 to 7 8 to 12	None	9.67 8.68	1.28 1.23	0.090 0.100	Average 0.680	Average 0.320
13 to 20 21 to 28 29 to 36 37 to 44 45 to 53	Methyl testosterone	7.35 7.81 8.53 9.68 9.07	1.27 1.36 1.40 1.45 1.50	0.111 0.091 0.192 0.330 0.313	Average 0.610	Average 0.400
54 to 61 62 to 69	None	9.42 11.23	1.52 1.51	0.269 0.296	Average 0.745	Average 0.342

were given in 20 mgm. doses, 5 times daily. Exact duplicate tablets without hormone were used as placebos during the control periods.

RESULTS

Clinical. Both patients had been carefully followed for 4 years prior to the present experiment, without any appreciable alteration in their condition during the past 2 years. Both complained of weakness and lack of energy. The girl was bed-ridden, whereas the man was able to carry out only desk work, though capable of strenuous manual labor prior to the onset of his disease. After several weeks of methyl testosterone administration, both patients manifested a sense of well-being, greater muscular strength, and an increase in appetite. The man, who had had only an occasional erection and no ejaculation for at least 4 years, spontaneously noted a return of normal frequency of erections and had several nocturnal emissions, as well as a definite sense of libido. The girl, interestingly, began to discuss her "boy friends" and displayed a degree of spontaneous

activity quite gratifying to all who had observed her previous lethargy. On identical appearing placebo tablets, this improvement persisted for a month and then disappeared.

Physical changes with treatment were definite. Secondary sex hair, which had completely disappeared in both, reappeared in the axillae and pubic regions after 3 or 4 weeks, and increased for several weeks after the introduction of placebos. However, no seborrhea appeared, unlike the almost invariable occurrence of this condition in eunuchoids so treated (21). Biceps tone and size apparently increased in both, though the element of enthusiastic cooperation on the part of the patients cannot be ruled out. The girl, however, in the latter part of the treatment was able to get out of bed and stand unassisted to be weighed, which she had not done for 2 years. Blood pressure remained essentially unaffected in both. The size of the prostate doubled to one-half adult size in the man, the testes were unaffected, and the uterus of the girl remained atrophic. Breast changes were borderline, if real, in both. Body

weight started to increase almost immediately, and continued to rise throughout the period of treatment, decreasing upon cessation of the drug. The subjects gained about 10 lbs. each during the limited period of treatment, the girl's weight increasing from 123 to 131 lbs., and the man's from 176 to 186 lbs.

**Laboratory.** Clear-cut changes occurred from the laboratory standpoint, also. The appearance of nitrogen retention and an increase in creatinuria were the most interesting, and are detailed below (Figures 1 and 2; (Table I). The basal metabolic rate tended to rise and the fasting serum cholesterol to fall, the cholesterol reaching unusually low levels for both (Table II). A slight rise in serum albumin and decline in globulin were suggested, but more data are needed. No change occurred in serum sodium, NPN, urinary 17-ketosteroid output, serum electrolyte partition including sodium, or in the complete blood count.

Oral glucose tolerance tests reverted to a normal pattern.

The composition of the diets was calculated from standard tables and was not analyzed. Stools were analyzed for nitrogen in both patients, with and without treatment, and the results in both periods were essentially the same. The urinary nitrogen output was about 3 grams greater daily in the woman without treatment than with it, and about 1 gram greater in the man. This difference was less toward the end of the treatment period in the latter. A loss of some of the retained nitrogen occurred in the man on stopping the drug. The administration of the drug decreased the wide daily fluctuations in nitrogen excretion.

A mild creatinuria was present during the control periods but became greatly exaggerated after 3 weeks of treatment. This slowly declined following cessation of the drug for about the same length of time. Renewal of therapy then raised

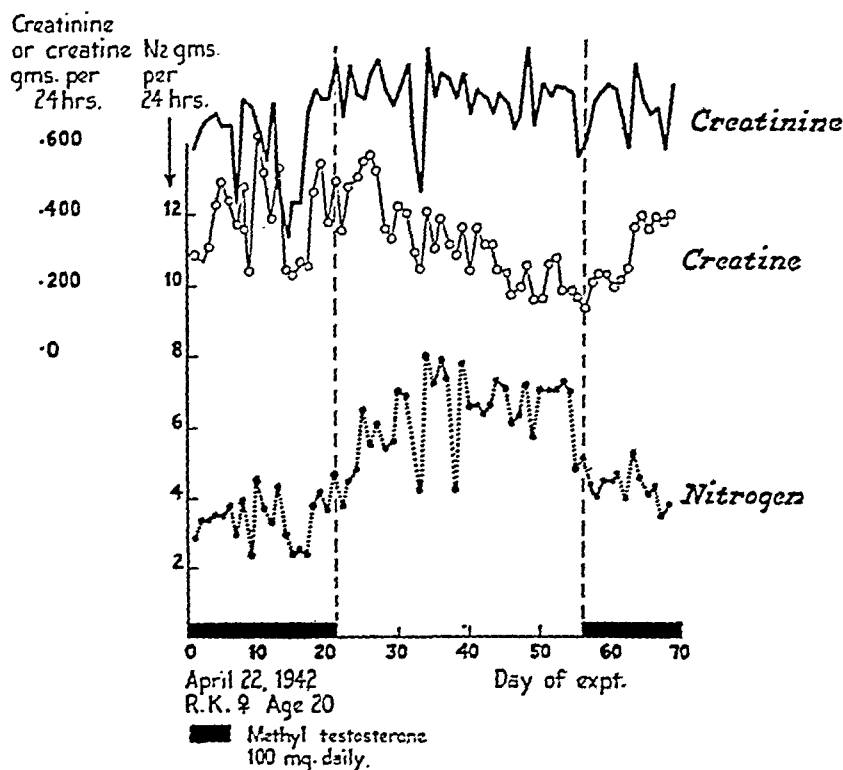


FIG. 1. GRAPH SHOWING THE EFFECT OF METHYL TESTOSTERONE THERAPY UPON THE URINARY EXCRETION OF NITROGEN, CREATINE, AND CREATININE OF PATIENT R. K., FEMALE, AGE 20, WITH SIMMONDS' DISEASE DUE TO AN INTRACRANIAL TUMOR

TABLE I

*Table listing the urinary excretion values for nitrogen, creatine, creatinine, inorganic phosphorus, and uric acid in Simmonds' disease, before, during, and after methyl testosterone administration*

*Patient R. K., female, on a diet containing 8.8 grams of protein N per day, less 0.4 grams N in stool*

Day of experiment. Start—April 21, 1942	Treatment	Nitrogen	Creatinine	Creatine	Phosphorus	Uric acid
		<i>grams per 24 hours</i>		<i>mgm. per 24 hours</i>		
1 to 7	Methyl testosterone	3.35	0.617	0.369	Average 0.460	Average 0.216
8 to 15		3.60	0.600	0.433		
16 to 22		3.19	0.560	0.354		
23 to 30	None	5.12	0.765	0.493	Average 0.620	Average 0.306
31 to 38		6.11	0.706	0.343		
39 to 46		6.82	0.744	0.317		
47 to 54		6.71	0.696	0.244		
55 to 62		6.53	0.708	0.196		
63 to 70	Methyl testosterone	4.47	0.693	0.202	Average 0.490	Average 0.282
71 to 77		4.26	0.700	0.357		

*Patient McA., male, on a diet containing 12.0 grams protein N per day, less 1.1 grams N in stool*

Start—May 1, 1942						
1 to 7	None	9.67	1.28	0.090	Average 0.680	Average 0.320
8 to 12		8.68	1.23	0.100		
13 to 20	Methyl testosterone	7.35	1.27	0.111	Average 0.610	Average 0.400
21 to 28		7.81	1.36	0.091		
29 to 36		8.53	1.40	0.192		
37 to 44		9.68	1.45	0.330		
45 to 53		9.07	1.50	0.313		
54 to 61	None	9.42	1.52	0.269	Average 0.745	Average 0.342
62 to 69		11.23	1.51	0.296		

were given in 20 mgm. doses, 5 times daily. Exact duplicate tablets without hormone were used as placebos during the control periods.

## RESULTS

**Clinical.** Both patients had been carefully followed for 4 years prior to the present experiment, without any appreciable alteration in their condition during the past 2 years. Both complained of weakness and lack of energy. The girl was bed-ridden, whereas the man was able to carry out only desk work, though capable of strenuous manual labor prior to the onset of his disease. After several weeks of methyl testosterone administration, both patients manifested a sense of well-being, greater muscular strength, and an increase in appetite. The man, who had had only an occasional erection and no ejaculation for at least 4 years, spontaneously noted a return of normal frequency of erections and had several nocturnal emissions, as well as a definite sense of libido. The girl, interestingly, began to discuss her "boy friends" and displayed a degree of spontaneous

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A mild creatinuria was present during the control periods but became greatly exaggerated after 3 weeks of treatment. This slowly declined following cessation of the drug for about the same length of time. Renewal of therapy then raised

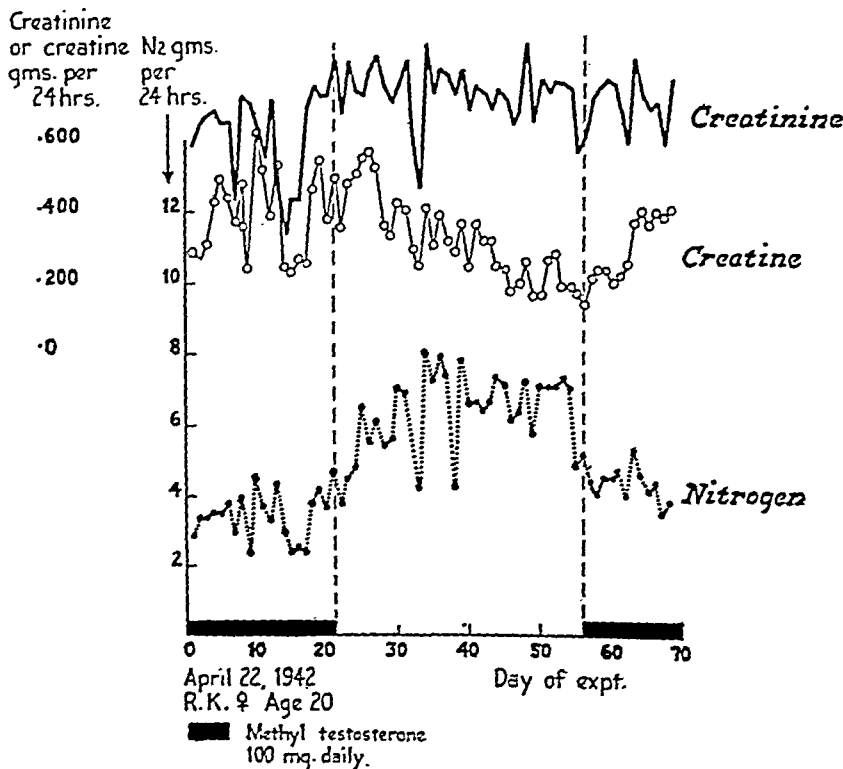


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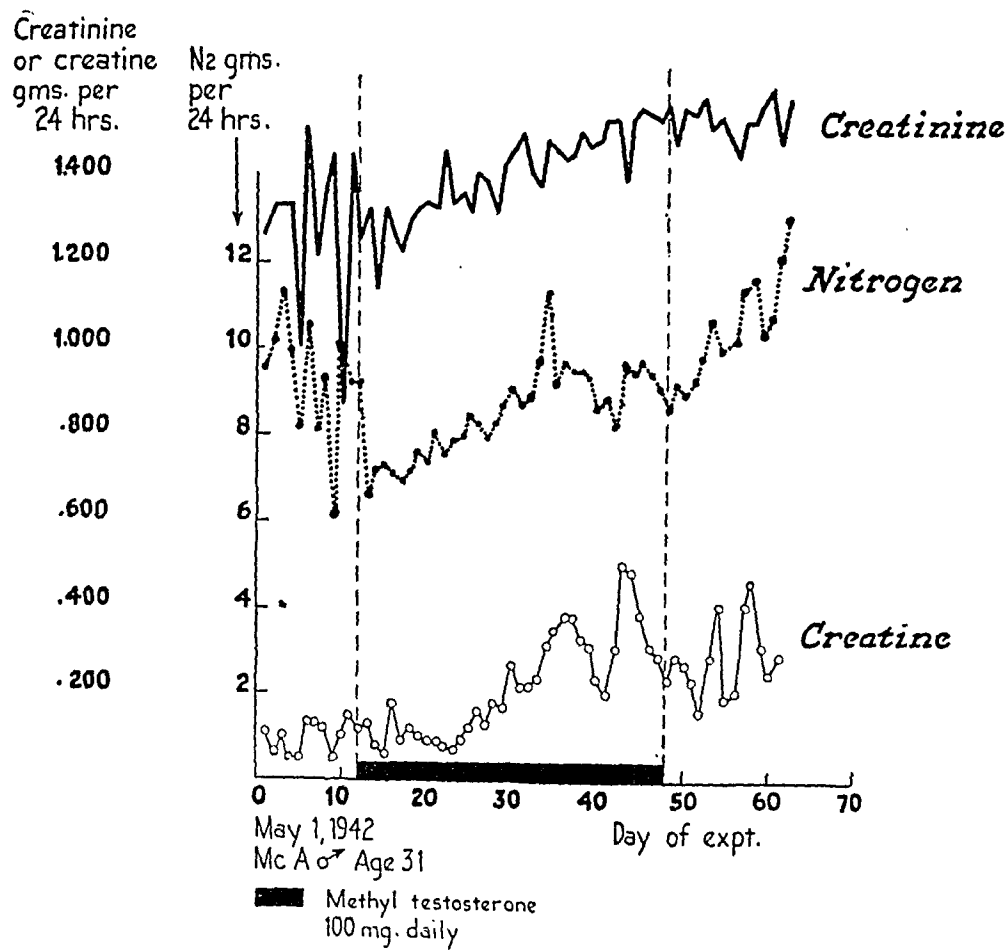


FIG. 2. GRAPH SHOWING THE EFFECT OF METHYL TESTOSTERONE THERAPY UPON THE URINARY EXCRETION OF NITROGEN, CREATINE, AND CREATININE OF PATIENT MCA., MALE, AGE 31, WITH SIMMONDS' DISEASE DUE TO A PITUITARY ADENOMA

TABLE II

Table showing the effect of methyl testosterone administration upon basal metabolic rate and fasting serum cholesterol in Simmonds' disease

McA.				R. K.				E. S.			
Treatment	Date	Basal metabolic rate	Fasting serum cholesterol	Treatment	Date	Basal metabolic rate	Fasting serum cholesterol	Treatment	Date	Basal metabolic rate	Fasting serum cholesterol
None	April 7, 1942	-34	187	None	March 20, 1942	-28	255	None	April 7, 1942	-40	328
	April 30, 1942	-41			April 8, 1942	-33			June 2, 1942	-23	288
	May 12, 1942	-36	204	Methyl testosterone	April 16, 1942	-46		None	July 21, 1942	-23	361
Methyl testosterone	June 11, 1942	-32	134	March 28, 1942 to May 13, 1942	April 23, 1942	-37	290	W. L. G.			
May 12, 1942 to June 17, 1942	June 17, 1942		107		April 30, 1942	-37		Methyl testosterone	June 15, 1942	-12	274
None	June 22, 1942	-41	94		May 8, 1942	-21					
	June 24, 1942		125	None	May 13, 1942	-26		February 28, 1942, to June 15, 1942			
	July 1, 1942	-39	148		May 25, 1942	-35					
				Methyl testosterone	June 19, 1942	-32					
				June 18, 1942 to June 30, 1942	June 23, 1942	-37	230				
					June 25, 1942	-32					
					June 26, 1942	-30	194				
					June 30, 1942	-28					

the output to its previous high levels, much more quickly. Uric acid excretion showed no definite correlation with the other values. Inorganic phosphate output tended to follow nitrogen. The creatinine excretion was inconsistent, inasmuch as it tended to rise during treatment in the man, and after treatment in the woman.

#### COMMENT

The effects of replacement therapy in the hypophysectomized rat, treated with anterior hypophyseal implants and extracts, are well known (2). Nitrogen retention occurs with little change in the other urinary constituents, except salts and phosphorus which parallel nitrogen output (1). Likewise, the extremely important role played by the anterior hypophysis in the regulation of nitrogen metabolism as it relates to protein storage and breakdown in the intact animal has been made progressively clearer (22). The fact that methyl testosterone will cause nitrogen retention, and hence presumably protein synthesis, during markedly reduced or possibly absent hypophyseal function, just as it does in the normal and in eunuchoidism (23), would tend to indicate that the fundamental mechanisms involved in nitrogen metabolism may be influenced in the absence of the hypophysis. This is also indicated by the fact that when nutrition is forcibly maintained at its previous level, weight loss may be prevented with maintenance of body nitrogen, despite the other manifestations of anterior hypophyseal lack (24).

Two alternative and less likely possibilities present themselves. It is possible that methyl testosterone may activate remaining hypophyseal tissue to an increased degree of function. The failure of 17-ketosteroids to rise in the urine is against this. The sole source of these urinary steroids in women is the adrenal cortex, as shown by the disappearance of these substances consequent to the destruction of the adrenal in Addison's disease (25). In men, an additional fraction is derived from the testes due to the conversion of testosterone in the liver (26). Since the secretory activity of both the adrenal cortex and of the testis is largely dependent upon the anterior hypophysis (2), failure of the latter results in the disappearance of 17-ketosteroids from the urine of both sexes. Renewed adrenotropic or, in the male, gonadotropic

activity under these circumstances might be expected to cause a re-rise in steroid output. Thus the failure of the cases under discussion to demonstrate such a rise is evidence against increased hypophyseal activity. Since testosterone has a gonadotropic action in maintaining spermatogenesis in the hypophysectomized animal (27), another possibility is that methyl testosterone may imitate other hypophyseal hormonal effects, namely, upon nitrogen metabolism.

The effect on creatine metabolism is of a degree which indicates a specific action of the hormone. Transmethylation (28) alone, with the utilization of all the methyl groups contained in the 100 mgm. of methyl testosterone for the synthesis of creatine, would account for only 135 mgm. if used solely for this purpose. The 400 mgm. levels of excretion attained by the patients studied here, and an output of 1,200 mgm. in 24 hours in a eunuchoid on this dosage (29), show the reaction to be beyond the amounts accounted for by the possibility of transmethylation.

The effects on basal metabolic rate and cholesterol are similar to those reported in eunuchoidism treated with methyl testosterone (30). The mechanism and significance of these changes is unexplained.

The failure of seborrhea or folliculitis to develop, despite secondary sexual hair growth in these and in the other 2 cases with this condition similarly treated, is important. These manifestations occur almost universally in eunuchoids so treated (21). It thus appears likely that certain basic conditions are necessary before androgens can act upon the skin to produce seborrhea. This fundamental preparation of the skin is lacking as a direct, or indirect consequence of the hypophyseal failure. Hamilton (31) has quantitated sebum secretion with a fluorescent light after ether extraction of the skin, but these methods have not as yet been employed in the cases presented in this study.

The growth of secondary sex hair is interesting in view of the absence of adrenal androgens, both before and during therapy, as revealed by the 17-ketosteroid excretion. The effect of the methyl testosterone on the man's genital tract corresponds to the effectiveness of the hormone in the hypophysectomized animal (32). This is not true of

the woman since the uterus failed to respond (33). Matters of dosage and length of administration may enter into this.

The striking clinical improvement consequent to treatment merits a word of comment. Sense of strength and well-being are indefinables which cannot be measured. However, one's experience with these patients tends to lend weight to the response noted here, a result which has failed to materialize with the use of many other therapeutic agents. How long the hormone will continue to be effective is being observed. Libido has persisted in the 2 patients not included in this study, for the past 6 months, with progressive weight gain during this time, while on the drug.

The question of whether hypophyseal function is entirely absent in these cases is a cogent one. In the rat, the removal of nine-tenths of the gland produces no symptoms (2), but beyond this, the whole picture of insufficiency may develop despite remaining fragments of the gland. Thus, these patients may have small amounts of functioning tissue even though the clinical picture of hypophysectomy is complete,—namely, pallor, asthenia, total loss of secondary sex hair, loss of genital function, no 17-ketosteroid excretion, and low basal metabolic rate. As for weight loss, the man was well below his former average weight of 205 lbs., but the girl was force-fed at home and was slightly obese. However, recent experience has shown that mild obesity may be present despite complete destruction of the anterior hypophysis, proven at autopsy (34). There is one feature in the girl which suggests possible hypophyseal activity, namely, a slight but definite increase in the size of the thyroid gland over the past few years. However, her basal rate remains — 40 per cent.<sup>3</sup>

Clinical results from the treatment of 2 other men with anterior hypopituitarism due to tumor were obtained during this study. The results, as far as libido, weight gain, and sense of well-being, have been as striking as in the cases reported here. Likewise, seborrhea failed to appear, as mentioned above, although growth of secondary sex characteristics occurred. Laboratory data are insufficient for presentation. Their case histories are appended.

<sup>3</sup> Subsequent autopsy failed to reveal any pituitary cells whatsoever.

## SUMMARY

Striking subjective, objective, and laboratory changes followed the treatment with methyl testosterone of 4 patients with Simmonds' disease.

Clinically, the patients demonstrated renewed vigor, sense of strength and libido, and redeveloped secondary sex characteristics.

Seborrhea failed to appear, suggesting that androgens act to produce seborrhea only after the skin has been prepared by some basic mechanism or mechanisms, which are wanting following hypophyseal failure.

In the absence of hypophyseal function, the administration of methyl testosterone resulted in nitrogen retention, associated with persistent weight gain.

Marked creatinuria developed after a latent period of several weeks, and subsided in about the same length of time after stopping treatment.

The therapeutic value of methyl testosterone in Simmonds' disease or anterior pituitary insufficiency is suggested by the cases in this series. A more extensive clinical trial of the drug is warranted, and more clinical and laboratory data should be accumulated.

The authors are indebted to Miss Genevieve Corbett and to Miss Elizabeth Zabriskie for technical assistance.

## CASE HISTORIES

Case 1. R. K. P. H. Unit No. 497847. Female. Single. Age 20 years.

The complete history to 1940 has been previously summarized (35). The diagnosis of aneurysm of the internal carotid artery within the skull was made, and wiring with electrothermic coagulation was performed in 1938, following the failure of bilateral ligation of the carotid arteries in the neck to halt the destruction within the skull. A true picture of advanced hypopituitarism, or Simmonds' disease, was present, due to the progressive destruction of the sella turcica and its contents. During the 2 years from 1938 to 1940, no further change within the skull occurred. There was some alleviation of the Simmonds' picture. A weight gain of 20 lbs. followed intensive feeding at home. A suggestion of pubic and axillary hair growth, and a glucose tolerance curve which tended to be diabetic in character, rather than the earlier flat curve, were noted. Urinary 17-ketosteroids remained zero.

In May 1941, a new set of symptoms appeared, consisting of spells of confused consciousness, preceded by flashes of light, incontinence, and alternate flushing and blanching of the face. There were no convulsive movements. The attacks lasted several minutes. They came

irregularly, from several hours to several weeks apart. Fasting blood sugars during the attacks were 90 to 100 mgm. per cent. An electroencephalogram revealed a "pathological picture consistent with a number of pathological states including cerebral anemia plus a destructive lesion in the right frontal and temporal regions." These alterations have persisted to the present.

In November 1941, the patient was readmitted for the 11th time, because of 3 months of mild diarrhea which soon cleared.

Physical examination: Her blood pressure was 80/60. Her striking facial pallor was unchanged. The angles of the mouth showed fissuring, redness, and erosion of the skin. A slight suggestion of pubic and axillary hair was noted. Her status otherwise was unchanged from previous occasions. A neurological examination revealed a left lower facial palsy, hypesthesia of the right side of tongue and face, a right 5th motor weakness, and a deviation of the tongue to the left.

Laboratory: The essential finding on this admission was in the x-ray of skull. This revealed a remarkable increase in the destruction of the skull as compared to the films of 1 year before. The wire previously inserted, was displaced posteriorly, the base of the skull was further damaged, the lateral wall and roof of the right orbit were further eroded, and the left optic canal was now 1 cm. in diameter. Several urine specimens showed a creatine of 0.09 gram and creatinine of 1.113 grams per 24 hours.

Course: The increased destruction of the skull in the face of the wiring, suggested the possibility that a tumor such as a chordoma, instead of an aneurysm, might be present, though Drs. Pfeiffer and Dyke felt the x-ray picture was still consistent with one, or several, aneurysms. The patient was essentially bedridden. She remained *in status quo* until her next admission for methyl testosterone therapy.

12th admission, Presbyterian Hospital, Ward H Met.  
March 20 to June 6, 1942. Age 20.

The history and physical examinations were unchanged. Her weight was 123½ lbs.

Laboratory: X-rays of skull were unchanged since 4 months previously. Dr. C. Dyke of the Neurological Institute believed an aneurysm of the right side of the circle of Willis was still the most likely possibility.

Course: The patient was started on methyl testosterone, 20 mgm., 5 times a day, by mouth, batch no. 1LL 17 of Schering's Oreton—M, 1 week after admission, to see the effect on her weakness. Several urinary creatines were done 1 to 2 weeks after starting therapy, and indicated that a definite increase had occurred. A gain in weight of 8 lbs. was noted and suggested the possibility of either water, fat, or nitrogen retention. Accordingly, a balance study was planned, and the results are shown in the figures and tables. Data concerning other laboratory findings are listed in Table II. Methyl testosterone therapy was continued until an unquestionable clinical improvement was noted by all, a month after starting treatment. The patient sat unassisted. A slight bladder dribbling ceased. She became alert, interested in her surroundings, and slept

less. No seborrhea was noted, though, by May 11th, pubic and axillary hair were definitely growing, and fine hairs were visible over abdomen, chest, and upper lip. Her weight fell but re-rose to 131½ lbs. No uterus was palpable on gynecological examination. On May 13, the patient was started on placebo tablets without hormone, batch no. 2SP1. No especial change occurred for 5 days. Then the patient had her first central nervous system attack in several months. From this point on, the patient slowly declined to her initial state of inactivity. By June 18th, she had lost all ground previously gained. She was recommenced on methyl testosterone, batch no. 2LL3. A weight gain of several pounds occurred again. The experiment was halted June 20th, however, because of the increasing severity of her central nervous system attacks. Serum protein values done on entry showed a total of 7.5 per cent, albumin 4.7 per cent, and globulin 2.8 per cent, and did not change during therapy.

Autopsy on October 11, 1942, showed an aneurysm of the internal carotid artery, posterior communicating branch, with no demonstrable pituitary tissue either on gross or microscopic examination. Death followed an intracranial exploration. This procedure was a last resort measure to attempt to control the increasing central nervous system involvement.

Case 2. A. McA. P. H. Unit No. 555816. Single.  
Male. Age 31 years.

The patient was first admitted to the Neurological Institute in October 1932, at the age of 21, because of headache, diplopia, and loss of all vision but light in the left eye, for the past year. This followed 3 years of increasing size of head, hands, and feet. There had always been some prognathism. The main findings on physical examination revealed early acromegalic features, generalized weakness, a temporal visual field defect in the right eye, and only upper left quadrant vision remaining in the left. His height was 6 feet and 1 inch, and weight 195 lbs. Laboratory work-up revealed a distorted sella consistent with a slow-growing tumor; overgrown sinuses and prognathism of the jaw consistent with acromegaly; and x-rays of the feet showing tufting of the terminal phalanges.

Course: He was given an intensive course of radiotherapy, 1,500 r. Only slight bitemporal hemianopia remained on discharge. He then received a second course of 1,500 r. following discharge from the hospital.

In October 1938, he was admitted to the metabolism ward for study. There was a family history of the mother having died of lymphatic leukemia. Physical examination revealed rheumatic fever in childhood without sequelae. He had had Autumn hay fever since he was 6; a T. & A. in childhood; a bone cyst removed from the left femur in 1934. He now complained of weakness, weight loss from 214 lbs. 5 years ago, down to 180 lbs. at present, and loss of libido and potency except for rare erections. Nocturnal emissions had ceased. He also complained of "anemia," i.e., marked pallor of the face. Physical examination revealed a typical acromegalic with a marked pallor of the face. There was no facial, axil-

lary, or pubic hair. The left optic nerve was pale. He shaved 3 times a week. He had fainted 3 times in the last year. Shoe size had decreased, since 1932, from 10 to 9, and shoes from 11E to 10½D; hat size from 7⅞ to 7½. He was bothered by the cold and tired too readily. His blood pressure was 92/70. The testes were normal in size but soft and mushy. The prostate was impalpable. There was a scoliosis of the thoracic spine to the left.

Laboratory: The hemoglobin was 14.5 grams; red blood cells 4.86 million; white blood cells and differential normal. Fasting serum cholesterol 178 mgm. per cent. Calculated serum sodium 137.1 m.eq. Blood NPN 32 mgm. per cent. Glucose tolerance tests on 2 occasions, with 100 grams of glucose orally, were similar. Fasting 68 mgm. per cent, ½ hour 60 mgm. per cent, not rising above 80 mgm. per cent. Kline blood test negative.

Course: The patient was followed in the clinic with little change. He was readmitted in April 1942 for a trial on methyl testosterone.

2d Admission, Ward G Met. April 30 to July 7, 1942.

The chief symptoms were still muscle weakness, inability to lift heavy objects or run up a flight of stairs, dyspnea on 2 flights of stairs, and constant sense of fatigue. He shaved once a week. There had been no ejaculation since the onset of the present illness though an erection occurred about once a week. There was no libido. Cramps in the muscles of the fingers and hands had been present for the past 7 to 8 months.

Physical examination was unchanged from the last admission and the blood pressure varied between 90/65 to 100/70.

Laboratory: Basal metabolic rate was 36 per cent. X-ray of skull unchanged. Fasting serum cholesterol 204 mgm. per cent. Blood sugar 83 mgm. per cent. Blood NPN 26 mgm. per cent. Calculated serum sodium 142.8 m.eq. Serum proteins 6.4 per cent with albumin 4.4 per cent and globulin 2.0 per cent.

Course: The patient was observed for 2 weeks without any therapy. During this period, he left the hospital between 9:30 a.m. and 5:00 p.m. to go to work, taking lunch with him from the hospital. He saved his urine during this time also. He was then given methyl testosterone, 20 mgm. 5 times a day, continuing the same regime of work and diet, etc. Within 2 weeks, he noted increased ability to work and walk stairs, and noted much less fatigue. There was a return of libido and an increase in the frequency of erections. After a month of therapy (June 17), the prostate had increased to ½ normal adult size. Pubic and axillary hair were growing, but no seborrhea was noted. Laboratory findings are recorded in Figure 1 and in Tables I and II. The serum protein was 6.2 per cent with albumin 4.6 per cent, globulin 1.6 per cent. For the last 2 weeks of the experiment, the patient was then started on an identical placebo pill without subjective change, except for some decline in strength. Three months after leaving the hospital, December 19, 1942, while taking methyl testosterone, 10 mgm. 5 times a day, he had gained up to 205 lbs., felt "the best he ever

felt in his life," and contemplated marriage. No seborrhea was noted though hair growth continued. The prostate was unchanged.

Case 3. W. L. G. P. P. Married. Male. Age 39 years. (Referred through the courtesy of Dr. Paul Sheldon.)

This patient was first seen in October 1941, because of declining sexual potency for the past two years. Family history revealed the mother to have had diabetes for years. His past history was essentially non-contributory except for a tonsillectomy at 10 and an operation for a deviated nasal septum at 20. The patient had eaten a peculiar vegetarian diet between the ages of 13 and 25. He felt the diet had insufficient protein. However, he underwent a normal puberty from 16 to 18 and had a normal sex life including marriage at 27 years of age. There were no pregnancies despite efforts. At 25 years of age, the patient developed a persistent anemia, later diagnosed "pernicious anemia," for which he had been taking feosol, thyroid, and liver, to the present. Two years before being seen here, the patient had been to Mexico where he had a diarrhea which cleared shortly, although his stools were said to continue to show an excess of fat. About this time, there was a sudden loss of libido with a loss of hair over the body, axillary, and pubic regions. After a year and a half, there was a sudden loss of vision in the right eye, without headache or vomiting. Libido declined to the point where his wife threatened a divorce. His weight had fallen 20 lbs., to 155 lbs. There was a continuous lack of energy. Physical examination revealed a very pale man without sexual hair, a prostate that could barely be made out, slightly soft testes, and eye defects described by Dr. Truman Boyes: "An absolute right central and paracentral scotoma, a constriction of the superior nasal field of the right eye and nasally of the left eye." The rest of the examination was negative. B.P. 110/70.

Laboratory: Hemoglobin was 14.1 grams; red blood cells 4.4 million; white blood cells 6,000 with 49 polymorphonuclear cells. The basal metabolic rate was 24 per cent. Fasting serum cholesterol was 267 mgm. per cent. X-ray of skull (Dr. C. Dyke) showed a large pituitary fossa, with large frontal sinuses and thinning of the dorsum sellae consistent with an adenoma. Glucose tolerance curve, with 100 grams glucose orally, showed only a slight rise and fall over 3 hours.

Course: The patient was started on sublingual testosterone in propylene glycol and 10 per cent alcohol (36), 15 mgm. 3 times a day, from December 13 to February 28, 1942, without appreciable change. On February 28, methyl testosterone, 10 mgm. 3 times a day by mouth, was started. Within 3 weeks the patient complained of too frequent erections. He had gained 4 lbs. He was continued on this dosage, except for 1 month on 20 mgm. 5 times a day. By June 15, hair was growing on face, pubis, and axillae. The basal metabolic rate was 12 per cent. Fasting serum cholesterol 274 mgm. per cent. The patient was then returned to 10 mgm. 3 times a day August 5, 1942. The patient stated in a letter "I'm in

blooming good health, everyone says I look better than I ever have, and I now weigh 170 lbs."

Case 4. E. S. P. H. Unit No. 544056. Male. Married. Age 63 years.

This patient was 63 years old at the time of the present experiment. He had been seen in the Neurological Institute, Presbyterian Hospital, and Vanderbilt Clinic since April 1928 because of an adenoma of the anterior lobe of the pituitary with hypopituitarism.

On first admission to the Neurological Institute, April 12 to April 29, 1938, his chief complaint was blurred vision for one year. The family history was irrelevant. His past history revealed August hay fever for years, fractured left leg 9 years ago, pneumonia 5 years ago, varicose veins and hemorrhoids 5 years ago, and small gonads since puberty, though no disturbance in sexual function had been noted till 5 years ago, when impotence developed. His present illness began 1 year before admission with headaches and progressive visual disturbances, including diplopia and loss of temporal vision. Physical examination revealed a Fröhlich type individual with scant beard, female escutcheon, fat breasts and small genitals, the testes being approximately  $1 \times 1 \times 1$  cm. The only other positive findings were pallor of the optic discs and bi-temporal hemianopia. The blood pressure was 136/80.

Laboratory: The basal metabolic rate was 23 per cent. X-rays of the skull showed enlargement of the sella turcica with atrophy of the posterior clinoids and dorsum sellae. X-rays of the optic foramina revealed bilateral atrophy consistent with a pituitary adenoma (Dr. C. Dyke).

Course: The patient received 7 x-ray treatments with some improvement in the visual fields. Subsequently, the patient had a series of admissions to the Presbyterian Hospital and the Neurological Institute. First admission to the Presbyterian Hospital, June 3 to 23, 1938, was for lobar pneumonia RLL, organism undetermined. At this time, the diagnoses of multiple left renal calculi, prostatic calculi, and cystitis were made. The patient was then transferred to the Neurological Institute from June 29 to August 25, 1938, because of progressive loss of vision. After a second course of x-ray therapy, a right frontal craniotomy with removal of most of a large pituitary adenoma was performed by Dr. C. Masson, July 21, 1938. A third course of radiotherapy was given shortly after discharge from the Hospital.

Subsequent events were the following admissions to the Presbyterian Hospital: September 13 to October 6, 1938, for chronic pneumonitis and asthma; April 3 to 20, 1939, for an acute right mastoiditis due to hemolytic streptococcus for which a simple right mastoidectomy was performed. Erysipelas developed in the wound but patient recovered uneventfully after this. A circumcision for phimosis was performed January 13, 1941. From October 16 to November 9, 1941, the patient was admitted to the G. U. service, for a left nephrectomy for renal calculus and an infected hydronephrosis. In December 1941, the patient bumped the unprotected area of his head. He de-

veloped mental derangement for a few days, requiring admission to the Manhattan State Hospital.

On April 6, 1942, patient was seen in the Vanderbilt Clinic regarding admission during methyl testosterone therapy. He refused admission but was willing to be followed in the O. P. D. At that time, he weighed 152 lbs., had been without erections for 10 years, felt tired, weak, and below par, but could work as a doorman. Examination showed marked pallor of skin, a few axillary and pubic hairs, small penis and retractile testes  $1 \times 1 \times 1$  cm., but was otherwise negative.

Laboratory features are recorded in the tables. On April 20, methyl testosterone, 10 mgm. 5 times a day, was started. When next seen, June 2, he had frequent erections, definite libido, "felt better, stronger" than in years. On examination, the penis was larger, pubic and axillary hair was growing, and hair was appearing about nipples. The serum cholesterol had fallen and basal metabolic rate had risen. Urinary 17-ketosteroids were unchanged (Table II). Methyl testosterone was stopped, and the patient was not seen until July 21. At that time he wished to restart the drug because of decline in well-being. He was restarted on methyl testosterone 10 mgm. 5 times a day. On September 23, he stated he was the "best he had been in years." He now weighed 184 lbs. and boasted of his ability to "sling trunks," which he had been unable to do before. Sexually, his improvement was maintained.

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# THE MOTILITY OF THE SMALL INTESTINE IN SPRUE<sup>1</sup>

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An abnormal small-intestinal pattern has been demonstrated by roentgenologic studies in both the tropical and non-tropical variety of sprue (1 to 3). Generalized hypomotility is observed, and the barium moves along the gut in thick, sausage-shaped masses which are separated by intestinal areas, apparently in a state of spasm. Many of the bowel loops are widely dilated and often contain considerable gas. At the same time, the mucosal relief is strikingly altered in that the usual markings of the *valvulae conniventes* are thickened, widely separated, and, in advanced cases, completely obliterated. In an admirable review of this subject, Golden (4) has, however, reiterated the original contention of Snell and Camp (1) that the observed changes are not characteristic of sprue, but may occur in other chronic deficiency states and, at times, may appear as the result of emotional stresses.

In 2 patients with sprue, whom we repeatedly intubated over a period of 8 and 13 months, respectively, the small-intestinal motor activity was studied by means of the balloon-kymograph method, described by Ingelfinger and Abbott (5). Of the 2 patients, the first would be considered to have the tropical, the second, the non-tropical variety of sprue.

## CASE REPORTS

*Case 1.* W. C., a 36-year-old man, first noticed loose stools at the age of 21, shortly after he had been purser for 6 months on a boat sailing between New York and South America. Since that time, he had suffered from intermittent bouts of weight loss, distension, cramps, and frequent bowel movements, consisting of light stools never containing blood or pus. In the year before entry to this hospital, he had been subjected to 3 ileostomies in an effort to relieve his distension and cramps.

Physical examination on July 18, 1941, revealed an emaciated individual with extreme hypotonia of the entire musculature. A brown pigmentation covered the forehead, cheilosis was present, and the tongue appeared dark red and smooth. The abdomen was markedly distended and tympanitic. Both a proximal and a distal ileostomy

opened onto the abdominal wall, which was severely irritated by the constant discharge of intestinal contents. The skin of the legs appeared slightly scaly.

A biopsy of a mesenteric lymph node and of small-intestinal tissue, taken when the ileostomies were closed, revealed evidences of chronic inflammation and a brown pigment (hemofuscin), diffusely deposited throughout the intestinal musculature as well as the lymphoid tissue.

*Case 2.* W. J., a 45-year-old man of Portuguese descent, was discovered to have a severe macrocytic anemia and hypocalcemic tetany, 4 years before entry. Although he experienced fatigue and weight loss, his bowel movements were to the best of his knowledge rarely abnormal. He had always lived in northern latitudes. Physical examination on admission revealed a fairly well-developed man with flaring of the costal margins, markedly protruding abdomen, and "clubbing" of the fingers. Tympanites was not striking.

Results of significant laboratory studies in both these cases are listed in Table I.

## RESULTS

When the record obtained from the jejunum of a patient with sprue is compared with a normal jejunal record, striking and consistent differences are noted (Figure 1). Whereas the normal upper small intestine prevents the standard balloon from filling with more than 10 to 30 cc. of air under pressures averaging 20 cm. of water, the gut in sprue offers much less resistance to increases in the volume of the balloon, even when the pressures in the balloon system are below 10 cm. of water. In our 2 cases, this lack of intestinal resistance to distension was roughly proportional to the severity of the disease. Thus, in Case 1, a pressure of 5 to 9 cm. of water was at times sufficient to inflate the balloon with 75 cc. of air. When the standard balloon contains this much air, an error is introduced by the fact that some of the resistance to distension is effected by the elasticity of the balloon itself; but this error does not invalidate the results, since it tends to decrease rather than increase the balloon volume. In Case 2, where the patient had less severe manifestations of sprue, 45 cc. of air was maintained in the balloon by a pressure of 10 to 15 cm. of water.

<sup>1</sup> This study was supported in part by a grant from Brewer & Company, Inc., Worcester, Massachusetts.



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## RECORDS OF JEJUNAL ACTIVITY

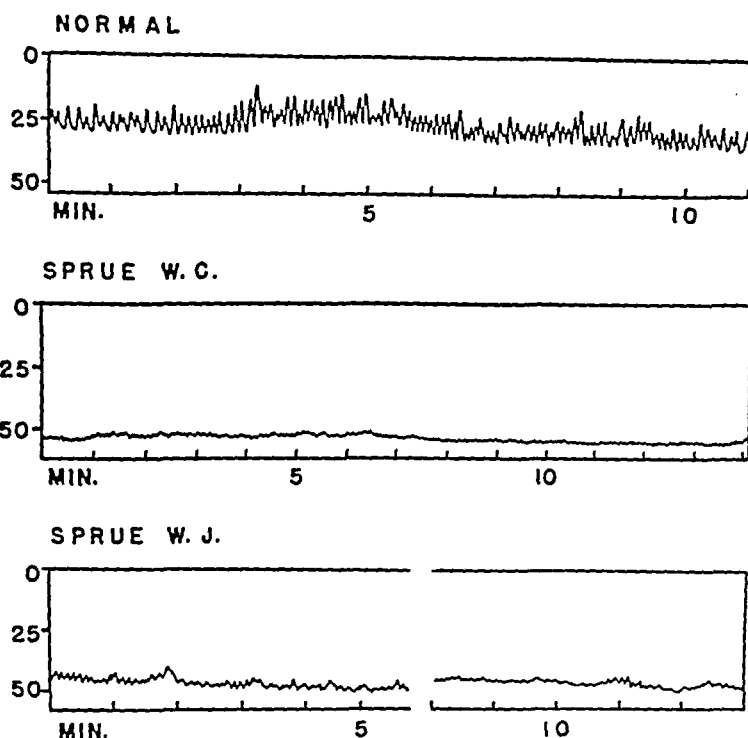


FIG. 1. COMPARISON OF THE RECORDS OF JEJUNAL ACTIVITY IN A NORMAL SUBJECT AND IN 2 PATIENTS WITH SPRUE

TABLE II  
*Vitamin medication given to 2 patients with sprue*

	July 18, 1941 to August 12, 1941	August 12, 1941 to October 3, 1941	October 4, 1941 to October 15, 1941	October 16, 1941 to November 12, 1941	November 13, 1941 to December 20, 1941	December 20, 1941 to January 12, 1942	January 13, 1942 to May 1, 1942	May 1, 1942 to June 2, 1942	June 2, 1942 to August 1, 1942
Vitamin A (USP units)	15,000	30,000	15,000	15,000	15,000	50,000 i.m.	30,000	15,000	30,000
Vitamin D (USP units)	2,500	5,000	2,500	2,500	2,500	5,000	5,000	2,500	5,000
Cevitic acid (mgm.)	75	75	75	75	75	75	75	50	50
Vitamin K (mgm.)	1 i.m.	1 i.m.	1 (twice a week, i.m.)	1 (twice a week, i.m.)	1 (twice a week, i.m.)	1 (per week, i.m.)	1 (per week, i.m.)	1 (per 2 weeks, i.m.)	1 (per week, i.m.)
Thiamin hydrochloride (mgm.)	10 i.m.	6	30 i.m.	0	0	0	0	0	0
Nicotinic acid (mgm.)	300	300	300	0	0	0	0	0	60
Liver extract (USP units)	0	8 (2 cc. twice a week for 13 doses, i.m.)	0	0	5 (5 cc. i.m.)	2½ (2½ cc. i.m.)	2½ (2½ cc. i.m.)	2½ (2½ cc. i.m.)	2½ (2½ cc. 2 times a week i.m.)
Brewer's yeast (grams)	2.5	0	0	0	0	0	0	0	0
Riboflavin (mgm.)	0	5	5	0	0	0	0	0	3
B complex* (cc.)	0	0	0	5 i.m.	5 i.m.	2½ i.m.	2½ i.m.	2½ i.m.	0

Vitamin medication, Case W. C. Unless otherwise noted daily and oral dosages are recorded.

\* Said to contain 3 mgm. thiamin hydrochloride, 10 mgm. nicotinic acid amide, 0.5 mgm. riboflavin, 0.450 mgm. pyridoxine, and 1.680 mgm. pantothenic acid per cc.

	January 21, 1942 to February 4, 1942	February 4, 1942 to February 27, 1942	February 27, 1942 to March 17, 1942	March 17, 1942 to April 4, 1942	April 4, 1942 to July 1, 1942
Brewer's yeast (grams)	0	50	0	0	0
Vitamin D (USP units)	4,000*	4,000*	4,000*	5,000	5,000
Vitamin K (mgm.) (oral preparation)	0	15*	0	0	0
Liver extract (USP units)	0	0	0	0	4 to 8
Vitamin A (USP units)	0	0	50,000 i.m.	30,000	(1 to 2 cc.) every 2 days † 30,000

Vitamin medication, Case W. J. Unless otherwise noted, daily and oral dosages are recorded.

\* The preparations used were said to be water-soluble.

† The patient was not regular in obtaining this medication.

TABLE I  
Results of significant laboratory studies in 2 patients with sprue

	Case 1	Case 2
<b>Hematological studies:</b>		
Red blood count (millions)	2.92	3.11
Hemoglobin (grams)	10.8	10.1
Hematocrit (per cent)	34.8	33.8
Mean corpuscular volume (c. $\mu$ )	119.0	109.0
Mean corpuscular hemoglobin ( $\gamma\gamma$ )	37.3	32.8
Mean corpuscular hemoglobin concentration (per cent)	31.3	30.2
Reticulocytes (per cent)	1.5	1.9
White blood count	5,000.0	8,000.0
<b>Stools:</b>		
Gross appearance	Yellow, unformed	Gray, formed
Guaiac test	1+	1+
Culture	Parasaccharomyces A	{No monilia or pathogenic organisms
<b>Fat content</b>		
Per cent of dried feces	56.0	49.0
Per cent of intake	80.0	20.0
<b>Blood chemistry:</b>		
Total protein (grams per 100 cc.)	5.4	7.1
Albumin (grams per 100 cc.)	4.6	3.8
Globulin (grams per 100 cc.)	0.8	3.3
Calcium (mgm. per 100 cc.)	8.0	7.3
Phosphorus (mgm. per 100 cc.)	2.0	2.7
Cholesterol (mgm. per 100 cc.)	65.0	125.0
Cholesterol esters (mgm. per 100 cc.)	50.0	90.0
Carotenoids (mgm. per 100 cc.)	less than 0.02	less than 0.02
Sodium (milliequivalents)	140.0	138.0
Prothrombin time (per cent of normal)	30.0	60.0
Phosphatase (King-Armstrong units)	5.0	10.0
<b>Glucose tolerance test:</b>		
Fasting level (mgm. per 100 cc.)	84.0	110.0
Peak rise (mgm. per 100 cc.)	111.0	90.0
<b>Vitamin A absorption:</b>		
Fasting level (I.U. per 100 cc. of plasma)	70.0	41.0
Peak rise	76.0	53.0
<b>Pancreatic enzymes in duodenal juices after stimulation with Mecholyl:</b>		
Amylase (Agren and Lagerlof)	560.0	322.0
Lipase (Crandall and Cherry)	18,230.0	22,560.0

The record of the normal upper small bowel is characterized by large (L) waves and small (S) waves, which may occur independently or may be superimposed. At the time that our patients were first studied, no spontaneous L waves were observed in Case 1 during 504 minutes of recording, and none in Case 2 during 57 minutes of recording. The S waves, while present and of a frequency lying within the normal range of 8 to 12 per minute, produced smaller excursions than are usually seen.

Although the most striking changes were observed in the jejunum, the records of duodenal and ileal activity in our patients showed similar deviations from normal.

The rate at which the balloon and tube were moved along the small intestine was only slightly slower than normal and occurred even during the period when L waves were not present.

#### RESULTS OF THERAPY

In our advanced case of sprue (Case 1), therapy affected the small-intestinal motor activity but slightly. No change at all was brought about by the daily parenteral administration of 30 mgm. of thiamin hydrochloride for 11 days or by the daily ingestion of 1.5 grams of choline hydrochloride for 1 week. Parenteral vitamin B complex and liver extract (see Table II) produced no improvement in the appearance of the intestinal

## RECORDS OF JEJUNAL ACTIVITY

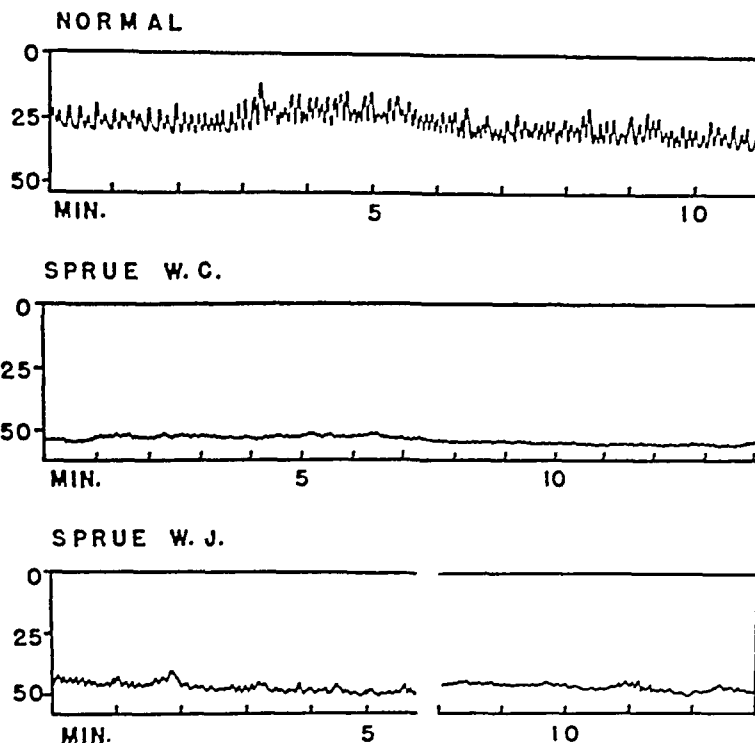


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Vitamin A (USP units)	15,000	30,000	15,000	15,000	15,000	50,000 i.m.	30,000	15,000	30,000
Vitamin D (USP units)	2,500	5,000	2,500	2,500	2,500	5,000	5,000	2,500	5,000
Cevitamic acid (mgm.)	75	75	75	75	75	75	75	50	50
Vitamin K (mgm.)	1 i.m.	1 i.m.	1	1	1	1	1	1	1
Thiamin hydrochloride (mgm.)	10 i.m.	6	(twice a week i.m.) 20 i.m.	(twice a week i.m.) 0	(twice a week i.m.) 0	(per week i.m.) 0	(per week i.m.) 0	(per 2 weeks i.m.) 0	(per week i.m.) 0
Nicotinic acid (mgm.)	300	300	300	0	0	0	0	0	60
Liver extract (USP units)	0	8	0	0	5	2½	2½	2½	2½
		(2 cc. twice a week for 13 doses i.m.)			(5 cc. i.m.)	(2½ cc. i.m.)	(2½ cc. i.m.)	(2½ cc. i.m.)	(2½ cc. 2 times a week i.m.)
Brewer's yeast (grams)	2.5	0	0	0	0	0	0	0	0
Riboflavin (mgm.)	0	5	5	0	0	0	0	0	3
B complex* (cc.)	0	0	0	5 i.m.	5 i.m.	2½ i.m.	2½ i.m.	2½ i.m.	0

Vitamin medication, Case W. C. Unless otherwise noted daily and oral dosages are recorded.

\* Said to contain 3 mgm. thiamin hydrochloride, 10 mgm. nicotinic acid amide, 0.5 mgm. riboflavin, 0.450 mgm. pyridoxine, and 1.680 mgm. pantothenic acid per cc.

	January 21, 1942 to February 4, 1942	February 4, 1942 to February 27, 1942	February 27, 1942 to March 17, 1942	March 17, 1942 to April 4, 1942	April 4, 1942 to July 1, 1942
Brewer's yeast (grams)	0	50	0	0	0
Vitamin D (USP units)	4,000*	4,000*	4,000*	5,000	5,000
Vitamin K (mgm.) (oral preparation)	0	15*	0	0	0
Liver extract (USP units)	0	0	0	0	4 to 8
Vitamin A (USP units)	0	0	50,000 i.m.	30,000	(1 to 2 cc.) every 2 days† 30,000

Vitamin medication, Case W. J. Unless otherwise noted, daily and oral dosages are recorded.

\* The preparations used were said to be water-soluble.

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JEJUNAL ACTIVITY AFTER TREATMENT

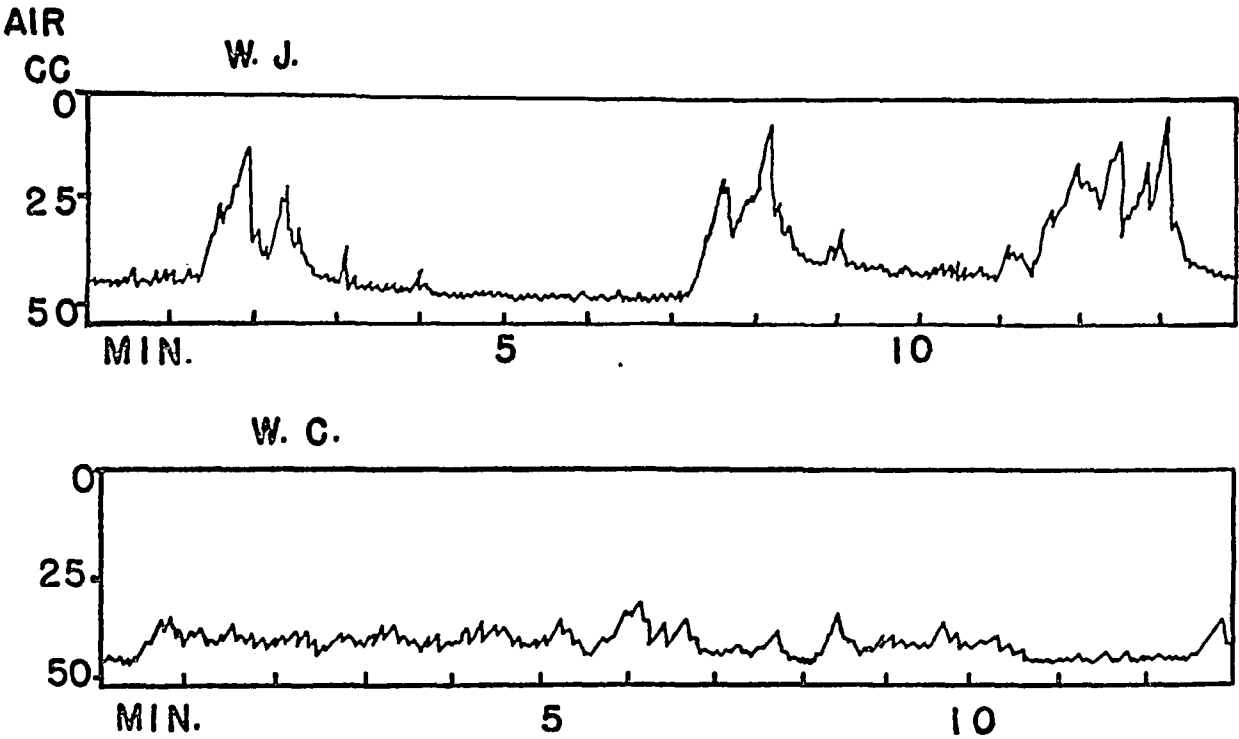


FIG. 2. RECORDS OF JEJUNAL MOTOR ACTIVITY IN 2 PATIENTS WITH SPRUE AFTER TREATMENT WITH PARENTERAL LIVER EXTRACT AND VITAMIN B COMPLEX

records during the early months of treatment; but after 8 months, a slight but definite return toward normal was recorded in the duodenum and high jejunum (Figure 2). The mid-jejunum, however, continued to show the same abnormalities which were exhibited before therapy was begun. Although the effects of therapy on intestinal motility were not remarkable, the clinical results were striking in that the patient gained 60 pounds, and

the stomatitis, distension, cramps, and diarrhea abated almost completely.

In Case 2, treatment with 50 grams of Brewer's yeast daily for 3½ weeks resulted in no change in the intestinal record. A record taken after the patient had received 2 to 4 cc. of liver extract every other day for 12 weeks showed striking L waves (Figure 2), which in this instance were associated with rapid advance of the balloon.

EFFECT OF CHANGING PRESSURES IN THE BALLOON  
(IN CM. OF WATER)

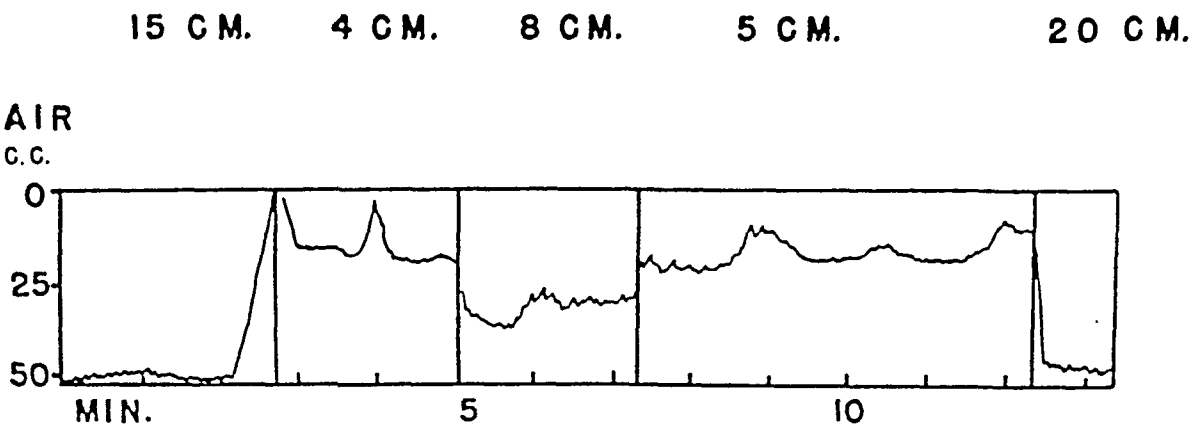


FIG. 3. DEMONSTRATION THAT THE SMALL INTESTINE IN SPRUE COMPRESSES OUR STANDARD BALLOON (50 CC. CAPACITY) WHEN IT CONTAINS ONLY 15 CC. OF AIR, PROVIDED THE PRESSURE WITHIN THE BALLOON IS SMALL

EFFECTS OF VARYING THE PRESSURES IN THE  
BALLOON SYSTEM

The pressures usually maintained in the balloon system (10 to 20 cm. of water) were reduced in order to determine whether the gut in severe sprue is in a constant state of relaxation, or whether the intestinal lumen can decrease in diameter if the intra-balloon pressures are minimal. As Figure 3 shows, a balloon inflated under a pressure of 5 cm. of water and containing only 15 cc. of air will occasionally be compressed by the intestinal activity. At other times, however, only respiratory movements can be recorded under similar conditions.

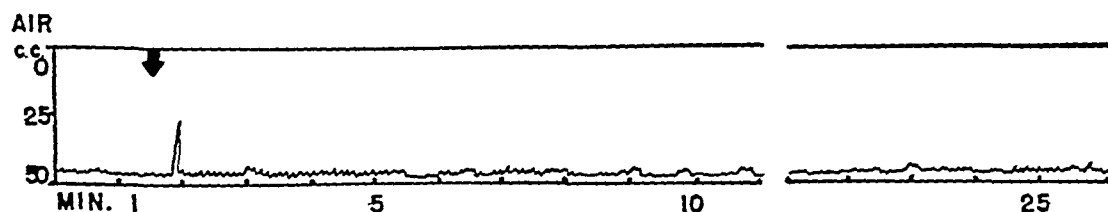
## EFFECTS OF DRUGS

The effects of prostigmine (0.5 mgm. subcutaneously), posterior-pituitary solution (20 units subcutaneously), and acetyl-beta-methylcholine chloride (10 mgm. subcutaneously) in Case 1 are shown in Figure 4. Each of these drugs was used

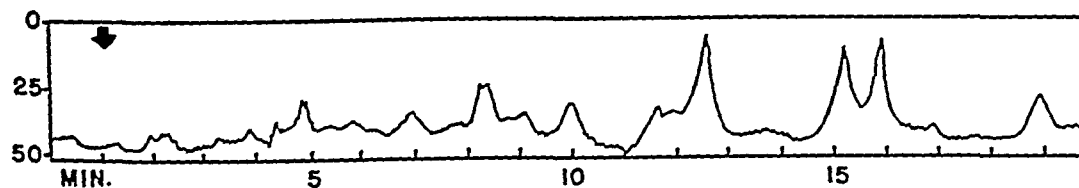
twice and the results can be summarized by saying that acetyl-beta-methylcholine stimulated, whereas prostigmine and posterior-pituitary solution did not affect the motor activity of the small intestine. The posterior-pituitary solution was, however, most effective in promoting the expulsion of gas and material from the large bowel, whose continuity with the small gut had been interrupted by an ileostomy. Desoxycorticosterone (5 mgm. intramuscularly on each of 3 consecutive days) produced no change in the small-intestinal motor activity, nor did 10 cc. of an aqueous extract of the adrenal cortex when given intramuscularly on each of 2 days.

In Case 2, prostigmine (0.5 mgm. subcutaneously) changed the control record only slightly, whereas acetyl-beta-methylcholine (10 mgm. subcutaneously) reduced the average balloon volume from 47 to 35 cc. In effecting this increase in the intestinal resistance to distension by the balloon,

## EFFECT OF SOL. POST. PITUITARY 1 CC. (20 UNITS) S. C.



## EFFECT OF ACETYL BETA METHYLCHOLINE 10 MG. S. C.



## EFFECT OF PROSTIGMIN 0.5 MG. S. C.

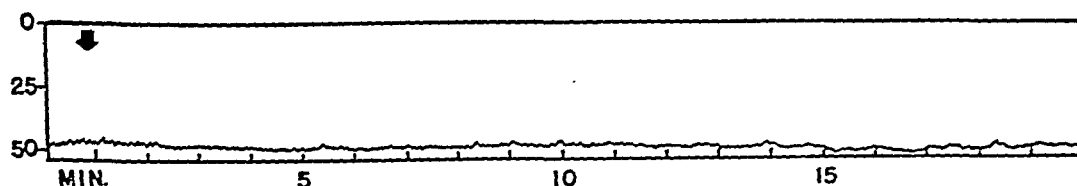


FIG. 4. EFFECT OF DRUGS ON THE SMALL-INTESTINAL MOTOR ACTIVITY IN A SEVERE CASE OF SPRUE

acetyl-beta-methylcholine produced an almost normal intestinal record for a period of 28 minutes.

In both patients, the effects of acetyl-beta-methylcholine on the intestinal record appeared 1 to 2 minutes after other effects of the drug, but persisted for 15 to 20 minutes after the profuse sweating and general feeling of discomfort had subsided.

#### DISCUSSION

Balloon records of intestinal motor activity are of significance only if correctly used and interpreted with due regard to the limitations of the method. There may be some question whether the presence of any balloon and tube in the intestines is physiologic; but if the diameter of the distended balloon exceeds the usual diameter of the gut, or if the balloon system produces intestinal obstruction by being anchored in one area—errors which are often encountered in the literature—the records can certainly not be considered indicative of normal intestinal activity. Further errors may be introduced by using large balloons in an organ of irregular outlines (such as the stomach), where a symmetrical balloon may at one time be affected by the actual intraluminal pressure of the organ, while at other times it is compressed by the walls of the hollow viscus. Furthermore, as Quigley and his associates (6) have pointed out, the large balloons usually employed, whether elastic or of relatively fixed volume, do not measure intraluminal pressure, but merely indicate intestinal activity by changes in the volume or the pressure within the balloon.

In our experiments, we have used an elastic balloon which can accommodate itself to changes in the intestinal lumen. This balloon is kept as closely as possible to a standard size so that it contains 50 cc. when filled, but not distended, with air. In this state, its diameter measures 3.5 cm. The balloon is inflated by pressures ranging usually from 10 to 20 cm. of water. These pressures are within the range of normal intra-intestinal pressures (6, 7). The balloon and attached tube are allowed to advance in accordance with intestinal activity by keeping plenty of slack in the stomach. Fluoroscopy is used to check the position of the tube.

The small-intestinal records obtained by this method show that the volume of the balloon in 2

patients with sprue is 2 to 3 times greater than when the balloon is inflated under similar pressures in the normal gut. The intestine in sprue can then be considered to lack tone, in the sense that the normal resistance to distension by intestinal contents is greatly diminished. This lack of tone is, so far as can be told from observation on only 2 cases, roughly proportional to the clinical severity of the disease.

The configuration of the S and L waves in the normal small intestine roughly corresponds to the Type I and Type II waves described in the human colon by Adler, Atkinson, and Ivy (8), although the frequency of the small waves in the small bowel appears to be greater than that in the colon. In our patients with sprue, L waves were conspicuously absent for long periods of time. When the balloons were inflated under slight pressures, however, or after stimulation with Mecholyl, or after a period of treatment, L waves were recorded. We may thus assume that L waves can occur in sprue under certain conditions, although their appearance is intermittent, and the intervals between their appearance may be lengthy. Since the L waves are most effective in advancing the balloon system and the intestinal contents trapped behind it, their intermittent appearance in a bowel lacking tone may account for the sausage-like clumping of barium sulfate observed in roentgenological studies of the bowel in sprue.

The S waves appear to occur in sprue as in the normal gut. Although comparable to the Type I waves seen in the colon, we do not believe that the S waves can be called non-propulsive, for the balloon can advance in the intestine even when L waves are not observed, and, as has been noted (5, 9), the S waves in the small intestine appear to travel down the gut.

Pendergrass and his associates (10) found that the roentgenologic appearance of the small intestine was altered by an olive-oil meal to such an extent that the pattern might be mistaken for that seen in steatorrhea. This finding led Snell (11) to suggest that possibly malabsorption of fat is the sole responsible factor in the production of the roentgenologic changes seen in sprue. So far as our records are concerned, however, this possibility can be dismissed, since our tracings were taken in intestinal segments empty of all food residues. Preliminary observations in sub-

ects with no gastrointestinal disease have also shown that the intra-enteric injection of olive oil may alter some of the motor activity of the small bowel, but never to the extent that the resultant changes might be confused with sprue.

Our records show that therapy with vitamins, particularly the B complex, may be followed by marked clinical improvement without correspondingly great changes toward normal in the tracings of intestinal motility. This observation differs from the roentgenologic findings of Snell (11) and of Miller and Rhoads (12), who noted marked improvement in the appearance of the small intestine after 4 days of treatment with liver extract, but it corresponds to the experience of Golden (4), who "has not seen this [that is, a complete return to normal] happen in a case of well-developed steatorrhea." In all likelihood, both the degree and the duration of the patient's illness determine whether or not therapy will be effective in restoring intestinal motor activity to normal.

In the normal small intestine, prostigmine increases the intestinal tone (as defined previously) and the frequency of the L waves (13). In our patients with sprue, prostigmine had little effect. Acetyl-beta-methylcholine chloride, on the other hand, appeared to stimulate intestinal motility in sprue as it does in normal individuals (14). Prostigmine is said to exert its cholinergic effect by inhibiting the choline esterase which normally destroys acetylcholine, whereas acetyl-beta-methylcholine chloride acts directly on the effector cells, as does acetylcholine (15). In view of these facts, our observations suggest that the primary defect in sprue is the inability of the intestinal autonomic nervous system to liberate acetylcholine. This hypothesis would explain the ineffectiveness of prostigmine, for the inhibition of choline esterase would increase intestinal motility only if acetylcholine were being produced. It is also consistent with the damage to the intramural nerve cells which Golden (4) has described.

A posterior-pituitary preparation (pitressin) was found by Elsom, Glenn, and Drossner (16) to increase the intestinal motor activity, particularly peristalsis. This effect was more pronounced in the more distal portion of the gut. Until the action of principles from the posterior-pituitary preparation upon the intestines is more fully understood, we cannot make any deductions

from the fact that these principles failed to affect the small gut of 1 patient with severe sprue.

#### SUMMARY

Records of the small-intestinal motor activity were repeatedly taken in 2 patients with sprue.

These records show that the gut in sprue lacks the usual resistance to distension exhibited by the normal intestine, that L waves are only intermittently present, and that S waves tend to be of small amplitude.

Treatment over short periods of time with individual fractions of the vitamin B complex produced little change in the tracings. Prolonged treatment with whole vitamin B complex produced some improvement in the records, but this improvement was not commensurate with the clinical results obtained.

Injections of acetyl-beta-methylcholine chloride stimulated the intestinal motility in our patients, but prostigmine was without effect. In 1 patient, posterior-pituitary solution, adrenal-cortical extract, and desoxycorticosterone produced no changes. These observations suggest that in sprue the nervous apparatus of the small intestine fails to liberate active acetylcholine.

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# INVESTIGATIONS OF MENINGOCOCCAL INFECTION. I. BACTERIOLOGICAL ASPECTS<sup>1</sup>

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Since the early part of this century, the literature dealing with the meningococcus and meningococcal infection has included intensive studies of the clinical, laboratory, and epidemiological aspects of the subject (1). Much of this early work was carried out during and after the last war, under the combined stimulus of wartime epidemics and the development of serum therapy. There followed a period of several years during which epidemics of meningococcal meningitis were uncommon, and investigations of the organism were limited to a comparatively few laboratories. In recent years, particularly since the advent of the present war, general interest in meningococcal infection has been reawakened and its potential importance among the infectious diseases has received new appreciation.

Despite extensive work with the meningococcus, many fundamental problems remain unsolved. Little is known, for example, regarding the nature of the factors which lead to the development of meningitis, to meningococcemia without meningitis, to the symptomless carrier state, and to no infection whatever. Much also remains to be learned regarding the specific biological characteristics which determine the pathogenicity of meningococci, the means by which the infected host is enabled to combat infection, the mechanism of natural immunity to the meningococcus, and the mechanism and durability of acquired immunity. The eventual solution of such problems will require a considerable amount of new data, which can only be obtained and correlated through clinical, bacteriological, immunological, and epidemiological methods of investigation.

In the hope of contributing further information in these directions, concurrent studies employing

these methods have been undertaken in a group of 42 cases of epidemic and endemic meningococcal infection. Both organisms and sera were available for study from most of these cases. Some were sporadic cases in the city of Boston, while others were seen during an epidemic of meningococcal meningitis in Halifax, Nova Scotia. Certain clinical and epidemiological aspects of the epidemic cases have already been reported (2, 3), and a more detailed study of the other cases will be the subject of a later report. All degrees of severity of illness were observed, ranging from mild, almost abortive infections, to the most fulminating type. In the majority of cases, the disease was moderately severe. All except 6 of the patients were adults. Two of the patients died; the rest recovered without any permanent sequelae. Most of the patients responded promptly to chemotherapy with either sulfadiazine or sulfapyridine. The temperature dropped during the first 2 or 3 days and symptomatic recovery occurred by the end of the first week. Only 3 of the patients were treated with therapeutic antiserum in addition to chemotherapy. In addition to these 42 cases, 15 carriers of meningococci and 60 normal contacts have also been studied.

The present report is concerned with the results of bacteriological investigation of this material; the immunological aspects have been considered separately (4, 5). The purposes of the bacteriological study have been (1) to examine certain general characteristics of all the strains of meningococci isolated, such as serological reactions, morphology, virulence for mice, and survival or growth in fresh normal serum, and (2) to compare, on the basis of these characteristics, the organisms isolated from the cerebrospinal fluid of cases and those from the nasopharynx of cases and carriers.

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<sup>2</sup> Fellow of the Frederick Tilney Memorial Fund.

## METHODS

Thirty-nine strains of meningococci have been investigated, of which 25 were isolated during the Halifax epidemic and 14 were obtained in Boston; 20 of these 39 strains were from the cerebrospinal fluid of cases, 4 from the nasopharynx of cases, and 15 from the nasopharynx of contact carriers.

The meningococci from the cerebrospinal fluid and nasopharynx were isolated either on casein-hydrolysate-starch agar (6) or on blood agar, employing a candle jar. Blood cultures were made in beef-heart infusion broth. When cultures from the patients were made after chemotherapy had been instituted, para-aminobenzoic acid was added to the media (7, 8).

As soon as each organism had been identified and typed, a 6-hour growth was suspended in milk and stored in a number of sealed tubes in a carbon dioxide-ice chamber. No more than 4 subcultures of any strain were made between the time of isolation and final storage. For the subsequent bacteriological studies, these stored organisms were cultured directly on solid media, then transferred once or twice before use; in a few instances, virulence titrations were made with freshly isolated strains before storage. In all of the tests, cultures grown for no more than 6 hours were used.

The following procedures were employed:

1. The organisms were grouped by means of *agglutination* in standard grouping sera; readings were made after 2 hours at 37.5° C. and 18 hours in the icebox. The meningococci have been designated as Group I (Types I and III of Gordon and Murray (9)) and Group II (Types II and IV). Also classed in Group II were a number of "atypical" carrier strains; these organisms fulfilled the cultural, morphological, and fermentative criteria for meningococci but typing by agglutination was inconclusive. Confirmation of the typing of the Groups I and II organisms was obtained through the kindness of Dr. Sara E. Branham, to whom samples of most of the strains were sent shortly after isolation.

2. *Quellung* (10) in specific antisera was used as an adjunct to grouping by agglutination.

3. *Fermentation reactions* were determined on agar media containing glucose, maltose, lactose, or sucrose. Solid media, containing phenol red as an indicator, were used.

4. Observations of the colonial and microscopic *morphology* of each strain were made and recorded.

5. The *oxidase test* (11) with para-aminophenylene-diamine was performed with all strains.

6. The *virulence* of each strain for mice was determined by the use of mucin. A single lot of mucin, which had been found to be satisfactory in preliminary titrations, was used in testing all the strains. A 3 per cent suspension in distilled water was prepared by mixing in a Waring Blendor. This mixture was then autoclaved for 15 minutes at 15 pounds pressure, and the reaction adjusted to pH 7.4. Fresh suspensions of mucin were prepared every 2 or 3 days.

The organisms were grown for 6 hours on serum-dextrose agar slants, then washed from the surface of the medium with beef-infusion broth and suspended in enough broth so that approximately 1,000,000,000 organisms were contained in each milliliter of suspension. The turbidity of the final suspension was kept as nearly the same as possible for all of the organisms by the use of a photoelectric turbidometer. Serial dilutions from  $10^{-1}$  to  $10^{-8}$  were made in broth. Plate counts of the  $10^{-5}$  to  $10^{-7}$  dilutions were made in triplicate, by spreading 0.1 ml. of the broth uniformly over the surface of a blood-agar plate. Although the experimental error inherent in this method is granted, triplicate plate counts were found to check with fair consistency, averaging 100 colonies from 0.1 ml. of the  $10^{-6}$  dilution. Although the assumption has been made for purposes of tabulation that each colony represents a single organism, it is realized that a colony may result from the growth of one or more organisms.

For the injection of mice, each broth dilution of  $10^{-3}$  and beyond was diluted 1:10 in mucin, so that final dilutions of  $10^{-4}$  to  $10^{-9}$  were obtained. These 6 dilutions were injected intraperitoneally into mice, in 1 ml. amounts. Six mice were used for each dilution. White Swiss mice from a single breed, weighing between 14 and 16 grams, were used in all of the titrations.

Four strains of meningococci were tested each day; these included 3 strains of unknown virulence and 1 virulent strain which was selected as a standard control for all of the titrations. The mice were observed for 72 hours after injection, although in most instances the fatalities occurred within 36 hours. Cultures of the heart's blood were made from at least half of the mice dying in each titration and, with rare exceptions, these were always positive for the meningococcus. The 50 per cent end-point for each titration was calculated by the method of Reed and Muench (12).

7. *The ability to survive or to grow in fresh normal human serum* was determined with 27 strains of meningococci from cases and carriers. These tests were made with the serum of a healthy adult male having a history of no previous meningococcal infection. Tenfold dilutions of young cultures of the organisms in broth were prepared, from  $10^{-1}$  to  $10^{-6}$ , and 0.05 ml. of each dilution was added to 0.25 ml. of serum diluted 1:2, in small glass tubes. The tubes were then sealed and rotated in the incubator for 20 hours, after which they were opened and cultures from each tube were made on the surface of a blood-agar plate. The original dilutions of the organisms were prepared so that the  $10^{-6}$  dilution contained approximately 50 organisms in 0.05 ml.

## RESULTS

1. *Grouping.* The distribution of specific groups among the Boston and Halifax cases and carriers is shown in Table I. In the Halifax epidemic, all of the strains from cases were Group I, 3 carrier strains were Group I, and 10 carrier strains were Group II, or atypical men-

TABLE I

*Distribution of specific groups in Halifax (epidemic) and Boston (endemic) strains of meningococci*

Source	Halifax strains		Boston strains	
	I	II	I	II
Cerebrospinal fluid of cases	9	0	6	5
Nasopharynx of cases	3	0	1	0
Nasopharynx of carriers	3	10	2	0

ingococci. In the Boston endemic cases, 7 strains were Group I and 5 were Group II. The 2 Boston carrier strains were Group I.

2. The *quellung* reaction was found to be of considerable value in the rapid grouping of fresh Group I meningococci. All of the strains which proved by agglutination to be Group I also exhibited capsular swelling in specific antisera when young cultures were used. It was found that this reaction could be enhanced to a notable degree if a small inoculum of organisms was allowed to grow for a few hours in heat-inactivated rabbit antiserum; by this method cultures grown for as long as 48 hours could be induced to show *quellung* after less than 4 hours' incubation in the antiserum. No *quellung* was observed with any of the Group II strains, although they were tested in several different lots of Group II antiserum.

3. *Fermentation reactions.* All of the strains produced fermentation of glucose and maltose, and failed to ferment sucrose or lactose.

4. *Morphology.* The colonial and microscopic morphology was similar in all of the Group I and Group II strains from cases, conforming to the classic descriptions of the meningococcus.

TABLE II

*Mucin virulence of meningococci for mice*

Source of strain	Group	Number of strains	Number of meningococci				
			1-100	101-500	501-1000	1001-10,000	Over 10,000
Cerebrospinal fluid	I	15	9*	2	2	1	1
	II	5	2	1		1	1
Nasopharynx	Cases I	4	1	2	1		
	II	0					
	Carriers I	5	4	1			
	II	10			1	3	6

\* The figures refer to the numbers of strains giving a 50 per cent end-point with the number of meningococci designated.

The colonies of 6 of the group II carrier strains from Halifax were smaller than usual on horse blood infusion-agar plates, and 2 of these had a faint yellowish pigmentation in older cultures.

5. *Oxidase test.* The colonies of all strains gave positive results with this test.

6. *Virulence.* The results of virulence titrations, expressed as the number of organisms constituting 50 per cent end-points, are shown in Table II. Strains with titers of 100 organisms or less are considered to be highly virulent. End-points of more than 1000 indicate moderate or questionable virulence, and those over 10,000 are assumed to be avirulent.

The Group I organisms, from both sporadic and epidemic cases, with 2 exceptions, were virulent. The exceptions were 2 strains of organisms of relatively low virulence from 2 sporadic cases in Boston. In such a small series of cases, this difference is of only speculative interest. The nasopharyngeal strains of Group I, whether derived from cases or carriers, were quite as virulent as the cerebrospinal fluid strains. Furthermore, in 3 cases from which both cerebrospinal fluid and nasopharyngeal organisms were isolated, no striking difference between the two strains was found. The titers of these strains are shown in Table III. Although in the case of J. C. there is ap-

TABLE III

*Comparison of mouse virulence of strains of Group I meningococci from the cerebrospinal fluid and nasopharynx of the same individuals*

Case	Number of organisms giving 50 per cent mortality	
	Cerebrospinal fluid strain	Nasopharyngeal strain
J. M.	7	10
B. R.	147	275
J. C.	11	352

parently considerable difference between the end-points for the nasopharyngeal and cerebrospinal fluid strains, the method of titrating virulence is probably not accurate enough to make this difference significant.

The few Group II strains from cases showed no striking differences from the Group I strains, as may be seen in Table II. Three were highly virulent, 1 was moderately virulent, and 1 was avirulent. In contrast, 9 of the 10 Group II carrier strains were of low virulence.

There was no correlation between virulence of the organisms, as determined by this method, and severity of the disease in patients from whom the organisms were isolated. The mildest case in the series, almost lacking in subjective symptoms, possessed one of the most virulent organisms, while another patient with extreme prostration and a stormy clinical course yielded an organism which was almost avirulent by mouse titration.

The storage of meningococci in the frozen state did not affect their virulence for mice. Several strains were tested after 8 months of storage and were found to be of the same order of virulence as at the time of original isolation. In contrast, two strains which had been maintained on blood agar for several months underwent a pronounced diminution in virulence. This diminution was accompanied by loss of the property of capsular swelling in specific antisera.

7. *Survival or growth in fresh normal human serum.* A wide variation was observed in the ability of different strains of meningococci to survive or to grow in fresh normal serum from a single individual. This property was not related to the virulence of the organisms for mice, nor to the specific group of the strain. The results with 12 strains isolated from cases of meningitis are shown in Table IV. It may be seen that all degrees of survival were exhibited by strains of the same mouse virulence and of the same group. The least virulent strain (Lu) and the most virulent strain (Number 4), for example, possessed

TABLE IV

*Ability of meningococci to survive in fresh, normal human serum in 1:2 dilution*

Strain	Group	Virulence *	Dilution of organisms †					
			10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
2	I	10	+	+	0	0	0	0
30	I	43	0	0	0	0	0	0
21	I	7	+	+	+	+	+	+
4	I	1	+	+	+	+	+	+
Lu	I	>10,000	+	+	+	+	+	+
Do	I	6	+	+	0	0	0	0
8	I	275	+	+	+	0	0	0
Ro	I	1200	+	+	+	+	+	+
Cz	II	18	+	+	+	+	0	0
Mc	II	80	+	+	0	0	0	0
Deo	II	500	+	+	+	+	+	+
Gi	II	9000	+	+	0	0	0	0

\* Virulence expressed as number of organisms producing 50 per cent mortality in mice, employing mucin.

† 10<sup>-6</sup> = 50 organisms in 0.05 ml.

equal ability to survive. On the other hand, 2 strains which failed to survive (Number 2 and Gi) had virulence titers of 10 and 9000, respectively. A similar variation was encountered among the other strains tested, in which were included 10 Group II carrier organisms of low virulence for mice. Some of the latter strains grew in the highest dilutions, while others failed to survive in any dilution.

When the nasopharyngeal and cerebrospinal fluid strains from 3 cases of Group I infection were compared, it was found that the nasopharyngeal strains were somewhat less able to survive in each instance (Table V). It should be noted,

TABLE V

*Comparison of the survival or growth in fresh, normal human serum of strains of Group I meningococci from cerebrospinal fluid and nasopharynx of the same individuals*

Case	Source of strain	Dilution of organism *					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
J. M.	Cerebrospinal fluid	+	+	+	+	+	+
	Nasopharynx	+	+	+	+	0	0
B. R.	Cerebrospinal fluid	+	+	+	+	+	0
	Nasopharynx	+	+	+	0	0	0
J. C.	Cerebrospinal fluid	+	+	+	+	+	+
	Nasopharynx	+	+	+	+	+	0

\* 10<sup>-6</sup> = 50 organisms in 0.05 ml.

however, that the differences were relatively small as compared with the degree of variation among cerebrospinal fluid strains from other cases.

## DISCUSSION

The bacteriological study of 39 strains of meningococci isolated under both epidemic and endemic conditions has revealed several points of interest. During the Halifax epidemic (2, 3), there were 93 cases within a period of 6 months. Unfortunately, the strains from all of these cases were not available for examination, but of the 12 strains tested in this laboratory, all were found to be Group I meningococci. Of the sporadic cases in Boston, 6 were due to Group I strains and 5 to Group II strains.

Single nasopharyngeal cultures of 60 contacts of 17 epidemic cases of meningitis yielded 3 Group I strains and 10 Group II strains. All

but 1 of the Group II carriers were found in a single household, comprising 17 members. No Group I carriers were found in this household. The observed incidence of Group I carriers in Halifax was low (5 per cent), possibly because only 1 culture was taken from each contact, and may not be indicative of the true carrier situation. Group I strains were isolated from the nasopharynx of 2 contacts of an endemic case of meningitis due to a Group I organism.

The morphology, fermentations, and oxidase reactions of these strains were found to be similar, regardless of their source and group. Minor differences in size of colonies were noted in 6 Group II carrier strains, and slight pigmentation developed with 2 of these strains.

The quellung reaction was found to be a reliable and satisfactory method of identifying the Group I strains, and in every instance was confirmed by the agglutination technique. It was also noted that the capsular reaction could be enhanced by permitting the organisms, before they were stained for examination, to grow for from 2 to 4 hours in rabbit antiserum, inactivated by heat. This procedure offers obvious advantages in grouping strains from the cerebrospinal fluid of patients. Another group of meningococci, termed II A (13), can be identified by the quellung reaction. Thus far, capsular swelling has not been described with the other, probably heterogeneous, strains comprising Group II.

Prior to the introduction of the use of mucin in virulence tests of the meningococcus by Miller (14), the estimation of virulence in mice required massive doses of organisms and the results did not permit easy and dependable comparisons between strains on a quantitative basis. With the use of mucin, relatively quantitative comparisons have become possible, and a number of workers have studied the virulence of different groups of meningococci, from cases, contact carriers, and non-contact carriers. If the virulence for mice could be taken as an index of pathogenicity for man, then differences of considerable degree might be expected in such comparisons.

Rake (15), in an extensive study of carrier strains of meningococci, found Group I strains to be of high virulence, Group II strains less virulent, and atypical strains very low in virulence.

In his experience, carrier strains of any given type tended to be of somewhat lower virulence than case strains of the same type. Silverthorne and his co-workers (16) reported that 90 per cent of the meningococci isolated from cases and contact carriers were virulent, while the majority of strains from non-contact carriers were avirulent. Most of the organisms studied by Silverthorne were Group II (75 out of 79). The Group II strains from carriers in the present studies were found to be non-virulent, but since these carriers were encountered during an epidemic of Group I meningococcal meningitis it seems reasonable to regard them as non-contact carriers. On the other hand, the Group I carrier strains were from contact carriers and were of the same virulence as the Group I strains from cases.

If mouse virulence indicates pathogenicity for man, these results would suggest that during epidemics, carriers are in some manner protected against the virulent organisms which they harbor, while non-contact carriers may have organisms which are simply accidental saprophytes, lacking in potential infectivity. In the paper following (4), it will be shown that Group I contact carriers possess relatively high titers of specific agglutinins, which are lacking in the sera of Group II carriers.

It has not yet been shown, however, that the mouse-virulence test actually constitutes a measure of pathogenicity for man. On theoretical grounds, the ability of a strain of meningococcus to survive or grow in normal human serum might also be taken as an indication of potential invasiveness, yet in this investigation the results of the two tests are at complete variance. It is conceivable that in both tests the results are determined by certain unrelated properties of the organisms which may contribute to the initiation of infection in man, but the problem is still unsolved. The ability of an organism to survive in a given normal serum will, of course, depend on the bactericidal action of that serum, which may or may not be due to specific antibodies. Variations between sera from different individuals would therefore be expected. Some property inherent in the organism, however, must be responsible for the observed results with different strains in the same serum. It is perhaps significant that

with strains from cases of meningitis, no direct correlation could be noted between either mouse virulence or survival in a single normal human serum and the severity of the clinical infection, the epidemic or endemic origin, or the serological group. There was also no apparent correlation between mouse virulence and source of the culture. Nasopharyngeal strains from 3 cases of meningitis, however, were somewhat less able to survive or grow in fresh normal human serum than were the cerebrospinal fluid strains from the same patients. Further study is necessary to determine whether or not this observation is of significance.

#### SUMMARY

1. Twelve strains of Group I meningococci were isolated from cases during an epidemic of meningitis. Seven Group I and 5 Group II strains were isolated from endemic cases. Nasopharyngeal cultures of a group of contacts in the epidemic area yielded 3 Group I strains and 10 Group II or atypical strains. Group I strains were isolated from 2 contacts of a sporadic case of Group I infection.

2. Quellung in specific antiserum was a satisfactory method for the rapid identification of Group I strains, and agreed in every instance with typing by agglutination. Quellung was found to be enhanced by culturing the organisms in specific antiserum for several hours. This reaction was not observed with the Group II strains.

3. The morphology, fermentations, and oxidase reaction were typical of meningococci in all of the 39 strains studied.

4. All except 2 of the Group I strains displayed a high virulence for mice. There was no significant difference between the virulence of nasopharyngeal and cerebrospinal fluid strains of Group I, between the Group I strains from cases and contact carriers, nor between the Group I strains from epidemic and sporadic cases.

5. Three of the 5 Group II strains from the cerebrospinal fluids of cases were highly virulent for mice, while 2 were of low virulence. In contrast, all of the carrier strains of Group II or atypical meningococci were of low virulence; these strains may represent non-contact carrier organisms, since they were isolated during a Group I epidemic.

6. No correlation was observed between the mouse virulence of strains from the cerebrospinal fluid of patients and the severity of the disease.

7. Wide variation was found in the ability of different strains of meningococci to survive or grow in fresh, normal, human serum, and no correlation was seen between the degree of survival and either the specific group of the organism or its virulence for mice. The nasopharyngeal strains of 3 cases were somewhat less able to survive than the cerebrospinal fluid strains from the same individuals.

The authors are indebted to Mrs. Muriel B. Stone and Miss Mary Glover for technical assistance.

Certain of the strains of meningococci and sera of patients used in this study were obtained during a survey of epidemic diseases in Halifax, Nova Scotia, undertaken by a group from Harvard Medical School, headed by Dr. J. Howard Mueller. The assistance and cooperation of Dr. A. R. Morton of the Office of Public Health and Welfare, Halifax, and of Dr. D. J. MacKenzie, Director of the Provincial Public Health Laboratory, are gratefully acknowledged.

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## INVESTIGATIONS OF MENINGOCOCCAL INFECTION. II. IMMUNOLOGICAL ASPECTS<sup>1</sup>

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The development of specific antibodies in human beings during recovery from meningococcal infection has been studied by a number of workers, who have employed a variety of methods, including agglutination (1 to 4), the opsonic reaction (5, 6), complement-fixation (7, 8), the bactericidal test (9, 10), and the mouse-protection test (11). Although immune bodies have been demonstrated with each of these tests during convalescence in some patients, the various reports show considerable irregularity in the results, especially with regard to the development of agglutinins and bactericidal antibodies. This irregularity may be due in part to differences between strains of meningococci used in the tests in different laboratories, as well as to differences in techniques. In some of the earlier investigations, the results were further modified by the use, in the majority of cases, of therapeutic antiserum. The nature of the immune response to meningococcal infection has therefore remained an unclarified problem.

It seemed possible that certain useful information might be secured by reinvestigating this problem in a group of patients, carriers, and normal contacts, by the concurrent use of a number of different methods for the estimation of specific antibody. The present study was undertaken in order to determine (1) to what extent and at what time, antibodies are produced following meningococcal infection; (2) by what serological tests such antibodies may most consistently be demonstrated; (3) what correlation, if any, exists between the development of antibodies and such factors as the severity of infection, the rapidity of recovery, or the biological characteristics of the infecting organism; and (4) what levels of anti-

body are to be found in the blood of carriers and of normal individuals in an epidemic area.

Thirty-four patients with Group I and 2 patients with Group II meningococcal meningitis were studied in detail. The clinical characteristics of some of these cases have been mentioned elsewhere (12). Serum was obtained at the time of admission to the hospital, which was usually within the first 2 or 3 days of the onset of the disease; another specimen was obtained during the second week and, in some cases, a third after several weeks. In the majority of cases, the temperature became normal within 2 or 3 days and convalescence was clinically apparent by the second week or earlier. Two deaths occurred. Sulfonamide drugs were used in the treatment of all of the patients; therapeutic antiserum was employed, in addition, in 3 cases of Group I infection. Serum was obtained from 4 Group I carriers, 6 Group II carriers, and 59 normal individuals with negative nasopharyngeal cultures who were in contact with cases during an epidemic of Group I meningococcal meningitis.

The following tests were employed in the study of these sera: agglutination, mouse-protection, quellung, plate precipitation (Petrie halo reaction (13)), complement-fixation, and the bactericidal test. Because of limited quantities of certain sera, it was impossible to perform each test with all specimens. In general, however, enough determinations were made in each instance to constitute a fairly representative group.

### AGGLUTINATION

#### *Method*

Several preliminary tests with homologous strains of Group I meningococci were carried out in the usual fashion, i.e., incubation for 2 hours at 37.5° C., followed by 18 hours in the icebox. By this method, agglutinins were detected in the convalescent serum of a number of patients. The titers were usually no higher than 1:16, how-

<sup>1</sup> This study was aided in part by a grant from the William W. Wellington Memorial Research Fund.

<sup>2</sup> Fellow of the Frederick Tilney Memorial Fund.

TABLE I

*Agglutination for Group I meningococcus by the serum of 10 patients during the acute and convalescent stage of the disease*

Case	J		G		E		Ro.		Fl.		H		Eg.		Ec.		Wo.*		D	
Day of disease	3	15	1	17	1	16	5	19	1	7	1	7	2	8	2	8	10	24	3	24
Agglutinin titer †	0	32	4	32	0	64	32	64	0	128	0	128	32	128	0	32	32	24	0	128

† Titer = highest dilution of serum producing definite agglutination.

0 = No agglutination in 1:4 dilution.

\* Early specimen of serum not available.

ever, and in order to obtain a more sensitive index of agglutination, a modification of the thread test (14) was employed. This consisted of the inoculation of a small number of organisms into serial dilutions of serum in a nutrient medium and the observation of agglutination with a hand lens during growth of the organisms. The medium in which the serum dilutions were made consisted of beef-heart infusion broth, containing 10 per cent rabbit serum, 2 per cent whole rabbit blood hemolyzed in an equal volume of distilled water, and 5 mgm. per cent of para-aminobenzoic acid. In tubes containing 0.25 ml. of this medium, serial twofold dilutions of the serum to be tested were made from 1:4 to 1:128. One-tenth ml. of a broth suspension, containing approximately 1,000 organisms, was added to each tube, after which they were incubated in a candle jar. Optimal agglutination was seen after from 10 to 12 hours' incubation. After 18 hours, agglutination was obscured by heavy growth of the organisms.

The sera of some patients were tested with strains of meningococci which had been cultured from their own cerebrospinal fluid, but it was found that the titers with these strains were the same as with a single stock strain of Group I meningococcus (strain No. 2). The latter strain was therefore used in tests with all of the sera from patients, contacts, and carriers. The sera of 2 patients with Group II meningococcal meningitis and of 6 Group II carriers were tested with homologous as well as with Group I organisms.

### Results

**Cases.** Of 34 convalescent sera from Group I cases (obtained during or after the second week), agglutinins for Group I meningococci were present in all except 1. In 23 of these cases, the acute serum (obtained on admission to the hospital) was also tested. Agglutinins were present in some of these acute sera, but a definite rise in titer between the acute and convalescent sera was found in all except 2 patients. This increase in titer is shown in Table I, in which the detailed results with the sera of 10 patients are shown. The distribution of agglutinin titers in 23 acute sera and 34 convalescent sera is shown in Table II.

The exact stage of the disease at which agglutinins first appeared is unknown, since the second

samples of sera were not obtained until at least the seventh day. Agglutinins were present in significant titer, however, in some patients at this time. In 1 patient (Table I, Ro.), the initial serum was obtained on the fifth day of the disease, and at this time, the agglutinin titer was 1:32. As is shown in Table II, 12 of 23 patients had

TABLE II

*Distribution of agglutinin titers in Group I patients and non-carrier contacts during an epidemic of Group I meningococcal meningitis*

Serum		Total number of persons	Serum dilution					
			0 to 1-4	1-8	1-16	1-32	1-64	1-128
Pa-tients *	Acute Convalescent	23	11	3	6	3		
		34	1	3	7	13	4	6
Non-carrier contacts	Total examined	59	33	7	12	4	3	
	Household A	6			3	2	1	
	Household B	5			3		2	
	Household C	9	9					

\* Acute—Serum obtained during first 2-3 days of disease.

Convalescent—Serum obtained during or after the second week.

titers of 1:8 or higher in the first serum taken during the first 3 days of the disease. It is not known whether these early agglutinins appeared as a rapid response to infection or were present before the onset of infection.

In 1 patient (Table I, Wo.), the agglutinin titer diminished from 1:32 on the tenth day to 1:8 on the twenty-fourth day. This, however, was the only instance in which a diminution in agglutinins was observed. In 10 other patients, there was no change in titer between the second and third weeks. The latest serum to be tested was obtained on the twenty-eighth day, and in this patient, there was no diminution in agglutinin titer. The only negative serum after the first week of the disease was that of an infant, aged 2 years, who had been ill for 3 weeks and who died of obstructive hydrocephalus several days later. An earlier serum from this patient was not available.

The acute and convalescent sera of 2 patients with Group II meningococcal meningitis were tested with homologous strains. No agglutinins were detectable in the acute sera, while both had titers of 1:16 after 2 weeks. One of these patients also possessed agglutinins for Group I organisms, which increased from 1:16 in the acute serum to 1:64 in the convalescent serum. On the other hand, no agglutinins for Group II organisms were present in the convalescent sera of 6 Group I patients who were tested.

**Contacts.** The sera of 59 presumably normal persons who were living in the homes of 12 cases of Group I meningitis were tested for agglutinins against Group I meningococci. A single nasopharyngeal culture in each of the contacts failed to reveal meningococci. The distribution of titers in these sera is shown in Table II. It may be seen that 40 were 1:8 or lower. When the individuals with titers of 1:16 or higher were grouped according to their place of residence, it was found that 11 were concentrated in 2 households. Group A was composed of 6 longshoremen in a waterfront rooming house in which a Group I carrier also lived. Group B consisted of 6 seamen who had been living together in quarters ashore. In contrast, 9 women and children contacts occupying household C had negative agglutination tests. The comparative titers in these 3 groups are shown in Table II.

**Carriers.** The sera of 4 carriers of Group I meningococci were found to have relatively high

agglutinin titers for Group I organisms (Table III). In contrast, 5 out of 6 Group II carriers had no detectable agglutinins for Group II organisms. The latter sera were also negative when tested with the Group I strain. One Group II carrier (A. D.) had agglutinins for Group I as well as for Group II organisms, in a titer of 1:16.

**Comment.** The agglutination test was found to be a useful method for demonstrating a rise in antibody during convalescence from meningococcal meningitis. It would appear, however, to be of little diagnostic value as far as active clinical infection is concerned because of the occurrence of appreciable levels of agglutinins in Group I carriers as well as in normal individuals. Furthermore, some of the patients possessed agglutinins early in the course of the disease and may have had them before the onset.

The findings in the group of normal contacts are of some epidemiological interest, in view of the concentration of persons with high agglutinin titers in the same households. Similar observations have been made by Finland and his co-workers (15, 16) in individuals exposed to cases of pneumonia and carriers of pneumococci. It is possible that these contacts may have been intermittent carriers, or may have had a clinically inapparent infection in the past. It is certain that they were repeatedly exposed to sources of meningococcal infection, in one instance a case and in the other a case and a carrier. The question arises as to whether such individuals as these are any less susceptible to meningitis than another group lacking agglutinins, as, for example, household C (Table II). In this regard, it may be noted that the patient who came from the longshoremen's rooming house (Table I, Eg.) had an agglutinin titer of 1:32 on the second day of his disease, which rose to 1:128 on the eighth day. The titer in the earlier serum is comparable with that in the other members of the household (Table II, A), and it is possible that this was the titer of the patient's serum before the onset of meningitis. If this is true, it would suggest that although repeated exposure may lead to the formation of agglutinins, this event does not confer immunity against meningitis. On the other hand, the presence of agglutinins early in the disease may merely mean that the day determined as the "first day of the disease" was not actually the first day of in-

TABLE III

*Agglutinins for homologous strains of meningococci in the sera of healthy carriers*

Group of carrier strain	Carrier	Serum dilution					
		1-4	1-8	1-16	1-32	1-64	1-128
I	D.	4*	4	4	3	2	2
	F.	4	4	4	4	4	3
	C.	4	4	3	2	1	0
	S.	4	4	3	2	1	0
II	M. L.	1	1	0	0	0	0
	M. H.	0	0	0	0	0	0
	N. H.	0	0	0	0	0	0
	B. H.	1	0	0	0	0	0
	L. L.	0	0	0	0	0	0
	A. D.	4	3	2	0	0	0

\* Figures refer to degree of agglutination:

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	S.	4	4	3	2	1	0
II	M. L.	1	1	0	0	0	0
	M. H.	0	0	0	0	0	0
	N. H.	0	0	0	0	0	0
	B. H.	1	0	0	0	0	0
	L. L.	0	0	0	0	0	0
	A. D.	4	3	2	0	0	0

\* Figures refer to degree of agglutination:

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Case	J		G		E		Ro.		Fl.		H		Eg.		Ec.		Wo.*		D	
Day of disease	3	15	1	17	1	16	5	19	1	7	1	7	2	8	2	8	10	24	3	24
Agglutinin titer †	0	32	4	32	0	64	32	64	0	128	0	128	32	128	0	32	32	24	0	128

† Titer = highest dilution of serum producing definite agglutination.

0 = No agglutination in 1:4 dilution.

\* Early specimen of serum not available.

ever, and in order to obtain a more sensitive index of agglutination, a modification of the thread test (14) was employed. This consisted of the inoculation of a small number of organisms into serial dilutions of serum in a nutrient medium and the observation of agglutination with a hand lens during growth of the organisms. The medium in which the serum dilutions were made consisted of beef-heart infusion broth, containing 10 per cent rabbit serum, 2 per cent whole rabbit blood hemolyzed in an equal volume of distilled water, and 5 mgm. per cent of para-aminobenzoic acid. In tubes containing 0.25 ml. of this medium, serial twofold dilutions of the serum to be tested were made from 1:4 to 1:128. One-tenth ml. of a broth suspension, containing approximately 1,000 organisms, was added to each tube, after which they were incubated in a candle jar. Optimal agglutination was seen after from 10 to 12 hours' incubation. After 18 hours, agglutination was obscured by heavy growth of the organisms.

The sera of some patients were tested with strains of meningococci which had been cultured from their own cerebrospinal fluid, but it was found that the titers with these strains were the same as with a single stock strain of Group I meningococcus (strain No. 2). The latter strain was therefore used in tests with all of the sera from patients, contacts, and carriers. The sera of 2 patients with Group II meningococcal meningitis and of 6 Group II carriers were tested with homologous as well as with Group I organisms.

### Results

**Cases.** Of 34 convalescent sera from Group I cases (obtained during or after the second week), agglutinins for Group I meningococci were present in all except 1. In 23 of these cases, the acute serum (obtained on admission to the hospital) was also tested. Agglutinins were present in some of these acute sera, but a definite rise in titer between the acute and convalescent sera was found in all except 2 patients. This increase in titer is shown in Table I, in which the detailed results with the sera of 10 patients are shown. The distribution of agglutinin titers in 23 acute sera and 34 convalescent sera is shown in Table II.

The exact stage of the disease at which agglutinins first appeared is unknown, since the second

samples of sera were not obtained until at least the seventh day. Agglutinins were present in significant titer, however, in some patients at this time. In 1 patient (Table I, Ro.), the initial serum was obtained on the fifth day of the disease, and at this time, the agglutinin titer was 1:32. As is shown in Table II, 12 of 23 patients had

TABLE II

*Distribution of agglutinin titers in Group I patients and non-carrier contacts during an epidemic of Group I meningococcal meningitis*

Serum		Total number of persons	Serum dilution					
			0 to 1-4	1-8	1-16	1-32	1-64	1-128
Pa-tients *	Acute Convalescent	23	11	3	6	3		
		34	1	3	7	13	4	6
Non-carrier contacts	Total examined	59	33	7	12	4	3	
	Household A	6			3	2	1	
	Household B	5			3		2	
	Household C	9	9					

\* Acute—Serum obtained during first 2-3 days of disease.

Convalescent—Serum obtained during or after the second week.

titers of 1:8 or higher in the first serum taken during the first 3 days of the disease. It is not known whether these early agglutinins appeared as a rapid response to infection or were present before the onset of infection.

In 1 patient (Table I, Wo.), the agglutinin titer diminished from 1:32 on the tenth day to 1:8 on the twenty-fourth day. This, however, was the only instance in which a diminution in agglutinins was observed. In 10 other patients, there was no change in titer between the second and third weeks. The latest serum to be tested was obtained on the twenty-eighth day, and in this patient, there was no diminution in agglutinin titer. The only negative serum after the first week of the disease was that of an infant, aged 2 years, who had been ill for 3 weeks and who died of obstructive hydrocephalus several days later. An earlier serum from this patient was not available.

The acute and convalescent sera of 2 patients with Group II meningococcal meningitis were tested with homologous strains. No agglutinins were detectable in the acute sera, while both had titers of 1:16 after 2 weeks. One of these patients also possessed agglutinins for Group I organisms, which increased from 1:16 in the acute serum to 1:64 in the convalescent serum. On the other hand, no agglutinins for Group II organisms were present in the convalescent sera of 6 Group I patients who were tested.

**Contacts.** The sera of 59 presumably normal persons who were living in the homes of 12 cases of Group I meningitis were tested for agglutinins against Group I meningococci. A single nasopharyngeal culture in each of the contacts failed to reveal meningococci. The distribution of titers in these sera is shown in Table II. It may be seen that 40 were 1:8 or lower. When the individuals with titers of 1:16 or higher were grouped according to their place of residence, it was found that 11 were concentrated in 2 households. Group A was composed of 6 longshoremen in a waterfront rooming house in which a Group I carrier also lived. Group B consisted of 6 seamen who had been living together in quarters ashore. In contrast, 9 women and children contacts occupying household C had negative agglutination tests. The comparative titers in these 3 groups are shown in Table II.

**Carriers.** The sera of 4 carriers of Group I meningococci were found to have relatively high

agglutinin titers for Group I organisms (Table III). In contrast, 5 out of 6 Group II carriers had no detectable agglutinins for Group II organisms. The latter sera were also negative when tested with the Group I strain. One Group II carrier (A. D.) had agglutinins for Group I as well as for Group II organisms, in a titer of 1:16.

**Comment.** The agglutination test was found to be a useful method for demonstrating a rise in antibody during convalescence from meningococcal meningitis. It would appear, however, to be of little diagnostic value as far as active clinical infection is concerned because of the occurrence of appreciable levels of agglutinins in Group I carriers as well as in normal individuals. Furthermore, some of the patients possessed agglutinins early in the course of the disease and may have had them before the onset.

The findings in the group of normal contacts are of some epidemiological interest, in view of the concentration of persons with high agglutinin titers in the same households. Similar observations have been made by Finland and his co-workers (15, 16) in individuals exposed to cases of pneumonia and carriers of pneumococci. It is possible that these contacts may have been intermittent carriers, or may have had a clinically inapparent infection in the past. It is certain that they were repeatedly exposed to sources of meningococcal infection, in one instance a case and in the other a case and a carrier. The question arises as to whether such individuals as these are any less susceptible to meningitis than another group lacking agglutinins, as, for example, household C (Table II). In this regard, it may be noted that the patient who came from the longshoremen's rooming house (Table I, Eg.) had an agglutinin titer of 1:32 on the second day of his disease, which rose to 1:128 on the eighth day. The titer in the earlier serum is comparable with that in the other members of the household (Table II, A), and it is possible that this was the titer of the patient's serum before the onset of meningitis. If this is true, it would suggest that although repeated exposure may lead to the formation of agglutinins, this event does not confer immunity against meningitis. On the other hand, the presence of agglutinins early in the disease may merely mean that the day determined as the "first day of the disease" was not actually the first day of in-

TABLE III

*Agglutinins for homologous strains of meningococci in the sera of healthy carriers*

Group of carrier strain	Carrier	Serum dilution					
		1-4	1-8	1-16	1-32	1-64	1-128
I	D.	4*	4	4	3	2	2
	F.	4	4	4	4	4	3
	C.	4	4	3	2	1	0
	S.	4	4	3	2	1	0
II	M. L.	1	1	0	0	0	0
	M. H.	0	0	0	0	0	0
	N. H.	0	0	0	0	0	0
	B. H.	1	0	0	0	0	0
	L. L.	0	0	0	0	0	0
	A. D.	4	3	2	0	0	0

\* Figures refer to degree of agglutination:

4—complete agglutination.

1—slight agglutination.

0—no agglutination.



fection. The nasopharynx may have been infected and bacteremia may have occurred some time before the clinical disease became severe enough to be recognized. Further studies along these lines seem to be indicated.

Although cross agglutination for Group II organisms was not noted in the convalescent serum of Group I cases, one of the patients with Group II meningitis developed agglutinins for both groups of organisms during convalescence. Also, a Group II carrier had agglutinins in a titer of 1:16 for Group I as well as for Group II organisms. It is possible that both of these individuals may previously have been exposed to Group I organisms.

#### MOUSE-PROTECTION TEST

##### *Method*

The serum to be tested, diluted 1:5, was injected intraperitoneally into mice in 0.5 ml. amounts, 30 minutes before the injection of organisms. The preparation of mucin suspensions was made in the same manner as that described for the virulence titrations in the preceding paper (17). White Swiss mice from a single breed, each weighing between 14 and 16 grams, were employed in all of the tests. A strain (No. 2) of Group I meningococcus was used which had been isolated from the cerebrospinal fluid of a patient; the virulence of this strain was such that 10 organisms produced 50 per cent mortality in mice. Each serum was tested for its protection against 100, 10,000, and 1,000,000 organisms, using 2 mice for each dose. Three groups of control mice were included with each test: one group received normal serum (1:5), the second, standard antimeningococcal serum (M19) of known protective action in 1:50 dilution,<sup>3</sup> and the third, no serum.

Tests were made with sera from 7 patients with Group I meningococcal meningitis and from 14 contacts and 2 Group I carriers in the Halifax epidemic. Since the sera of most of the patients contained sulfadiazine in varying amounts, para-aminobenzoic acid in a concentration of 5 mgm. per cent was added to all of the sera before injection.

##### *Results*

*Patients.* In tests with the sera of 7 patients, 2 showed no significant protection in either the acute or convalescent serum, 2 showed protection in both samples of serum against 100,000 50 per cent lethal doses, 2 showed protection against 100,000 and 1 against 1,000 50 per cent lethal doses in the convalescent serum.

<sup>3</sup> This standard antiserum was supplied through the courtesy of Dr. Sara E. Branham.

The 2 patients whose "acute" serum possessed protective action were known to have received sulfonamide therapy shortly before coming under our observation. It seemed possible that the apparent protection might be due to the presence of sulfadiazine in the serum. Because of this, separate experiments were undertaken to determine whether sulfadiazine could be inhibited *in vivo* by para-aminobenzoic acid, under the conditions of the mouse-protection test. The results of these experiments have been reported in detail elsewhere (18). In brief, it was found that good protection was provided by as little as 0.0025 mgm. of sulfadiazine per mouse, and that this effect was not inhibited by the presence of 10 mgm. per cent of para-aminobenzoic acid in the serum nor by a single subcutaneous injection of additional para-aminobenzoic acid at the time of the introduction of organisms. The protective action of sulfadiazine was eliminated only after repeated subcutaneous injections of para-aminobenzoic acid were made at 3-hour intervals. Presumably this was necessary to compensate for the rapid excretion of para-aminobenzoic acid (19).

The extremely small amounts of sulfadiazine required to induce protection of mice, and the difficulties involved in inhibiting this protection with para-aminobenzoic acid, render the mouse-protection test an unreliable method for assessing antibody in the sera of patients who have received sulfonamide therapy. False positive results occur when the concentration of sulfadiazine in serum is as low as 0.5 mgm. per cent. The test with patients' sera was therefore discontinued.

*Carriers.* One of the Group I carrier sera protected against 100,000 50 per cent lethal doses and the other against 10 50 per cent lethal doses.

*Contacts.* Seven contact sera possessed no protective action. Five showed slight or questionable protection. One protected against 100,000 and 1 against 10 50 per cent lethal doses.

These results with carrier and contact sera are shown in Table IV, in which the agglutinin titer of each serum for the Group I meningococcus is also indicated.

*Comment.* No conclusions can be drawn concerning the patients' sera in which protective action was demonstrated because of the possibility of interference by small amounts of sulfadiazine in the serum. The 2 negative results with convales-

TABLE IV

*Mouse protection tests with the sera of Group I carriers and contacts during an epidemic of Group I meningococcal meningitis*

Source of serum		Agglutination titer	Number of organisms injected		
			1,000,000	10,000	100
Carriers	F	1-128	2*	2	2
	S	1-32	0	1	2
Non-carrier contacts	McQ	1-32	2	2	2
	Cu	1-32	0	1	2
	T	1-64	0	0	1
	R	1-64	0	0	0
	Rd	1-32	0	0	0
	Pg	1-32	0	0	0
	K	0	0	0	0
	M	0	0	0	0
	McE	0	0	0	0
	D	0	0	0	0
	H	0	0	0	1
	Sw	0	0	0	1
	P	0	0	0	1
	McD	0	0	0	1
Controls	Normal serum	0	0	0	0
	No serum		0	0	0
	M-19 antiserum, dil. 1-50		2	2	2

\* Figures refer to the number of mice surviving with each dilution of organisms. Two mice were inoculated in each group.

cent sera, on the other hand, are valid indications of the absence of mouse-protective antibody in the dilution employed. One of these sera possessed agglutinins in a titer of 1:16 and the other in a titer of 1:32. Both were negative in the quellung and complement-fixation tests, to be reported subsequently. Both were obtained on the eighteenth day of the disease.

The sera of 2 Group I carriers and 2 contacts (McQ. and Cu.) in which protective antibody was detected also possessed relatively high titers of Group I agglutinins. Four contact sera with equal or higher agglutinins had no protective effect, however, so that no reciprocal correlation between agglutinins and mouse-protective antibodies is to be seen. It is of interest, nevertheless, that no protection was exhibited by any of the sera lacking agglutinins.

#### QUELLUNG

Capsular swelling of the Group I meningococcus is best observed when very young cultures are used, and is enhanced by prolonged contact of the

organisms with the antiserum. The standard method for demonstrating quellung, namely that of mixing a suspension of organisms with immune serum on a slide, has proved rather unsatisfactory on occasion because of the rapid loss of this property in older cultures. Some strains may exhibit good quellung in 6-hour cultures but none after 12 hours of growth. On the other hand, excellent capsular swelling occurs when the organisms are allowed to grow in specific antiserum for a few hours, regardless of the age of the culture at the time of inoculation.

#### Method

Each serum was inactivated by heating at 56° C. for 30 minutes. Para-aminobenzoic acid was added to the serum to make a concentration of 5 mgm. per cent. A single cerebrospinal fluid strain of Group I meningococcus was used in all of the tests. Of a 10<sup>-4</sup> broth dilution of this organism, prepared from a 6-hour serum-dextrose agar growth, 0.05 ml. was added to 0.25 ml. of the serum. The mixture was then incubated in a candle jar, and a loopful examined on a slide, with methylene blue stain, at hourly intervals.

It was found that the optimal time for demonstrating quellung was between 2 and 4 hours of incubation. Quellung was less marked when excessive growth occurred and, similarly, was less evident when excessive inoculum was employed.

The sera of 23 patients with Group I and 2 patients with Group II meningococcal meningitis were tested in this manner. The same sera were tested for quellung by the standard slide method. Tests were also made with the sera of 4 Group I carriers.

#### Results

The convalescent sera of 6 Group I patients produced definite quellung of the Group I meningococcus. The acute sera of these patients showed no quellung. Acute and convalescent sera from 17 other Group I cases and 2 Group II cases, and sera from the 4 Group I carriers, all yielded negative results.

When the 6 positive sera were tested for quellung by the slide method, using young cultures of the same Group I meningococcus which was employed in the incubation tests, 1 produced questionable capsular swelling and the remaining 5 were negative.

*Comment.* The 6 convalescent sera which produced quellung of the Group I meningococcus also contained agglutinins. Two had titers of 1:128, 3 of 1:64, and 1 of 1:32. Three of these patients

had received specific antiserum, while 3 were treated with chemotherapy alone. The degree of quellung was about the same in both groups. One patient's serum showed good quellung 2 days after the injection of antiserum, but none 1 month later.

The results indicate that although sera from the majority of patients with Group I infection do not show capsular swelling during convalescence, the test may be of some value in estimating antibody levels in the course of treatment with therapeutic antiserum. The incubation technique of demonstrating quellung is easily performed and appears to be considerably more sensitive than the usual slide method; when positive, it indicates that a high antibody level has been established.

#### PLATE PRECIPITATION TEST (PETRIE HALO REACTION (13))

##### *Method*

Beef-infusion agar plates were prepared so as to contain the serum to be tested in a dilution of 1:20. These were inoculated at single points with heavy scrapings from a plate culture of Group I meningococcus and incubated in a candle jar for 5 days. Control plates containing Group I antimeningococcal rabbit serum showed definite halo formation after 24 hours and very pronounced halos after 5 days.

##### *Results*

Two acute sera and 4 convalescent sera from patients with Group I meningococcal meningitis were tested. No precipitation was observed, although 2 of the convalescent sera possessed agglutinin titers of 1:64 in the thread test and also produced quellung of the Group I meningococcus. Both were positive in the complement-fixation test.

*Comment.* Because of limited quantities of convalescent serum, it was not possible to carry out plate precipitation tests with higher concentrations of serum. In view of the negative results with sera which possessed relatively high antibody titers by other methods, the plate precipitation method was discontinued. It is possible that positive results might have been obtained if higher concentrations of serum could have been used.

#### COMPLEMENT-FIXATION TEST

The convalescent sera of 26 patients with Group I meningococcal infection were tested for complement-fixing antibodies with an antigen prepared

from Group I organisms. The acute sera of 14 of these patients were also tested. Sera from 3 Group I carriers and from 30 normal contacts in the Halifax epidemic were examined. Two normal adults were injected with 25 ml. and 40 ml., respectively, of commercial Group I rabbit antiserum,<sup>4</sup> and complement fixation tests were made before and after these injections.

##### *Method*

The antigen was prepared according to a modification (20) of McNeil's method (21) for the preparation of gonococcus antigen. Six different strains of Group I meningococcus, isolated from spinal fluid and stored on carbon dioxide ice, were employed.

The procedure in the complement-fixation test was as follows: 0.25 ml. of varying dilutions of serum (inactivated by heating at 56° C. for 30 minutes) was mixed with 0.25 ml. of the antigen, diluted 1:40. Two units of complement contained in 0.5 ml. of normal saline were added, after which the mixtures were incubated for 30 minutes at 37.5° C. Five-tenths of a milliliter of 2 per cent sheep cells previously sensitized with two units of amboceptor was then added to each tube and again incubated for 30 minutes at 37.5° C. Appropriate anticomplementary and hemolytic controls were included in each titration. A positive rabbit antiserum of known titer (1-2048), and a normal human serum lacking in antibody, were run as controls with each test. The titer of the serum was regarded as the highest dilution producing definite fixation of complement. The results of the titrations have been expressed by the final dilution of serum employed.

##### *Results*

The results of the tests with the sera of patients, carriers, and contacts are summarized in Table V. It may be seen that none of the 14 sera from patients during the acute stage of the disease contained complement-fixing antibodies, while 16 of the 26 convalescent sera were positive. In the 14 patients whose acute as well as convalescent sera were tested, the actual development of antibodies was demonstrated in 8.

One of the 3 Group I carrier sera possessed antibody in a dilution of 1:32. The other 2 were negative. All of the 30 sera from normal contacts in the Halifax epidemic were negative in a final dilution of 1:4.

The majority of positive reactions occurred with convalescent sera obtained during the second week after the onset of the disease. In 2 pa-

<sup>4</sup> Furnished by Lederle Laboratories, Inc.

TABLE V

*Titers of complement-fixing antibodies for Group I meningococcus in sera of patients, carriers, and contacts*

Serum	Total number	Complement-fixation titer					
		<1-4	1-8	1-16	1-32	1-64	1-128
Patients acute	14	14					
Patients convalescent	26	10	2	5	6	2	1
Group I carriers	3	2			1		
Non-carrier contacts	30	30					

tients, a diminution in antibody titer was demonstrated later in convalescence. In 1 of these cases, the titer at 1 week was 1:16 and after 3 weeks, the test was negative. In the other case, the titer at 1 week was 1:256 and after 4 weeks, it had fallen to 1:64.

Three of the patients in this series were treated with therapeutic Group I antiserum. The sera of 2 of these patients were tested 3 days after the administration of antiserum, and the complement-fixation titer in each was 1:32. The third patient was tested 2 weeks after the administration of antiserum, and the test was negative at this time. In order to determine the increase in antibody titer which is produced by therapeutic antiserum, 2 normal adults were given antimeningococcal Group I rabbit serum intravenously. The first subject received 25 ml. of antiserum; after 20 minutes, the titer of his serum was 1:8. The second subject received a total of 40 ml. of antiserum in 3 divided doses at 2-hour intervals; 2 hours after the last dose, the titer was 1:64, and 18 hours later, it had fallen to 1:32. In the sera of both of these individuals, quellung of the Group I meningococcus was demonstrable by the incubation method following the injection of antiserum.

*Comment.* The results of the tests with passively immunized normal individuals suggest that the complement-fixation test as used here is not a highly sensitive index of specific antibody, since the titers are relatively low. There are, however, certain advantages in such a test. False or non-specific positive results seem less likely to occur, and a positive result assumes considerable significance. This is supported by the fact that in

the series of 73 sera tested, no positives were encountered except in convalescent patients and in 1 Group I carrier.

The titers in the convalescent sera may be correlated to some extent with the results of the agglutinin tests previously described. Of the 10 convalescent sera yielding negative results with the complement-fixation test, 9 were tested for agglutinins. Eight of the 9 sera had agglutinin titers of 1:32 or lower. On the other hand, of the 16 convalescent sera with positive complement-fixation tests, 12 had agglutinin titers of 1:32 or higher, and 5 of these had titers of 1:128. This correlation is shown in Table VI. It is not a

TABLE VI

*Relation between complement-fixing antibodies and agglutinins (thread reaction) for Group I meningococcus in convalescent patients' sera*

Complement fixation test	Number of sera	Titer of agglutinins				
		1-8	1-16	1-32	1-64	1-128
Negative	9	2	2	4		1
Positive	16	1	3	3	4	5

reciprocal relationship, however, since it may be seen that low agglutinin titers were present in some of the positive complement-fixation sera, while 1 of the negative sera had an agglutinin titer of 1:128. Furthermore, some of the contact and carrier sera, which showed no reaction in the complement-fixation test, had relatively high agglutinin titers.

#### BACTERICIDAL TEST

##### *Method*

The method was similar to that employed in previous bactericidal tests with the influenza bacillus (22). The organisms used in each test were grown for 6 hours on serum-dextrose agar slants and then suspended in beef-infusion broth from which serial dilutions from  $10^{-1}$  to  $10^{-8}$  were made in broth. The turbidity of the original suspension was adjusted by the use of a photoelectric turbidometer so that 0.1 ml. of the  $10^{-6}$  dilution contained approximately 100 organisms.

In testing the bactericidal action of fresh serum, 0.25 ml. of serum was mixed with 0.05 ml. of each dilution of organisms, in small Pyrex tubes. In other tests, the sera were inactivated by heating at  $56^{\circ}$  C. for 30 minutes. Dilutions of these sera, in 0.05 ml. amounts, were mixed with 0.25 ml. of fresh normal human serum, diluted 1:2, or with fresh normal defibrinated blood, and 0.05 ml. of each dilution of organisms was added. The tubes were

sealed and rotated in an incubator for 20 hours. After incubation, the contents of each tube were thoroughly mixed and two loopfuls were inoculated on the surface of a blood agar plate. Para-aminobenzoic acid in a final concentration of 5 mgm. per cent was added to the materials used in all tests in order to inhibit the effect of any sulfonamide in the sera.

### Results

Preliminary bactericidal tests with the convalescent sera of patients with Group I meningococcus infection yielded paradoxical results. It was found necessary to undertake a reinvestigation of the mechanism of bactericidal action in normal and immune sera before satisfactory tests could be performed with the sera of patients.

The results of this investigation have been reported in detail elsewhere (17, 23), but the observations which are pertinent to the present study may be summarized as follows: (1) It was found that Group I meningococci could be divided roughly into two classes—those which were susceptible and those which were resistant to the bactericidal action of fresh normal human serum, as indicated by their failure or ability to survive (17). Some variation was also encountered in the bactericidal property of sera from different normal individuals; for example, occasional sera were found in which the usually susceptible strains were able to survive. (2) No bactericidal action could be demonstrated in the fresh serum of rabbits which had been intensively immunized against Group I meningococci, even for strains which ordinarily were killed by normal rabbit serum. When these immune sera were diluted in the presence of normal rabbit complement, however, strong bactericidal action against resistant strains was produced by dilutions as high as 1:60,000, while a prozone was produced by dilutions of 1:600 or lower. Furthermore, when immune serum was added to fresh normal serum, inhibition of the normal bactericidal action for susceptible strains was observed, suggesting that a prozone might be responsible. (3) When fresh normal human serum was used as complement, with specific rabbit antisera or human convalescent sera, no killing of the resistant strains took place. When defibrinated blood was used instead of serum as complement, killing of these strains was caused by high dilutions of immune sera, suggesting that another mechanism, presumably phagocytic, was operative. (4) When fresh serum which lacked bactericidal action was employed as complement with the susceptible strains, killing was produced by immune sera.

In the light of these findings, tests were made with the sera of patients with Group I meningococcus infection, with the following results.

*Bactericidal effect of fresh serum.* The undiluted sera of 3 patients were tested for bactericidal action against the homologous organism during the acute and convalescent stages of the disease. In each instance, the serum was strongly bactericidal during the acute stage and definitely less bactericidal during convalescence (Table VII).

TABLE VII

*The bactericidal action of fresh serum for homologous organisms during the acute and convalescent stages of Group I meningococcus meningitis*

Patient	Day of disease	Dilution of organisms					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
R.	3	0	0	0	0	0	0
	14	+	+	+	+	+	+
E.	3	0	0	0	0	0	0
	11	+	+	0	0	0	0
L.	2	0	0	0	0	0	0
	14	+	+	+	+	+	+

0 = No growth.

+ = Growth.

*Effect of patients' sera upon bactericidal action of fresh normal serum.* These tests were carried out in the same way as the usual bactericidal test, except that the fresh normal serum used as complement was of itself bactericidal for the organisms. Of undiluted serum obtained during the acute and convalescent stages, 0.05 ml. was added to 0.25 ml. of normal serum, diluted 1:2. A susceptible strain (No. 2) was employed.

The sera of 7 patients produced no effect upon the bactericidal action of normal serum. The convalescent sera of 4 patients caused inhibition of bactericidal action, while the acute serum was without effect (Table VIII).

*Bactericidal effect of convalescent serum in the presence of neutral human complement.* When the susceptible strain (No. 2) was used with fresh serum from an individual lacking bactericidal action for this strain, the addition of convalescent serum produced striking results. Killing took

TABLE VIII

*The inhibitory effect of convalescent serum on the bactericidal action of fresh normal serum*

Patient	Day of disease	Dilution of organisms					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
R.	3	+	0	0	0	0	0
	14	+	+	+	+	0	0
E.	3	+	+	0	0	0	0
	11	+	+	+	+	+	+
J.	2	0	0	0	0	0	0
	14	+	+	+	0	0	0
Hu.	2	+	0	0	0	0	0
	14	+	+	+	0	0	0
Normal serum alone		+	0	0	0	0	0

0 = No growth.

+ = Growth.

place with 1:6,000 dilutions of the immune serum, while a prozone occurred with 1:6 dilutions of serum. In contrast, serum obtained during the acute stage had no effect (Table IX).

With a resistant strain (No. 21), human serum was found to be ineffective as complement. No bactericidal antibody was demonstrable either in convalescent serum or in rabbit antisera. When human defibrinated blood was used instead of serum as complement, however, the results were comparable with those obtained in the preceding test with the susceptible strain. Strong bactericidal action was caused by the convalescent sera of 4 patients, while the acute sera were negative.

TABLE IX

*Bactericidal effect of acute and convalescent sera for a susceptible strain of Group I meningococcus (No. 2) with non-bactericidal human serum as complement*

Day of disease	Dilution * of serum	Dilution of organisms					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
3	1-6	+	+	+	+	+	+
	1-60	+	+	+	+	+	0
	1-600	+	+	+	+	+	0
	1-6000	+	+	+	+	+	0
10	1-6	+	+	+	+	+	0
	1-60	0	0	0	0	0	0
	1-600	0	0	0	0	0	0
	1-6000	+	+	+	0	0	0
Complement alone		+	+	+	+	+	+

0 = No growth.

+ = Growth.

\* Figures indicate the final dilution of serum.

The results with the sera of 1 of these patients are shown in Table X.

*Bactericidal effect of sera from Group I carriers.* The sera of 2 Group I contact-carriers were tested with the resistant strain, using defibrinated blood as complement. Two samples of serum were obtained from each individual, one at the time of the first nasopharyngeal culture and another after 3 weeks, at which time cultures were negative in both. Both carriers had received a short course of sulfadiazine treatment during this 3-week interval. In one of the carriers, both samples of sera were negative. In the other, the first serum was negative but the second showed bactericidal antibody in a dilution of 1:60. It is of some interest that this individual underwent

TABLE X

*Bactericidal effect of acute and convalescent sera for a resistant strain of Group I meningococcus (No. 21) with normal human defibrinated blood as complement*

Day of disease	Dilution of serum *	Dilution of organisms					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
3	1-12	+	+	+	+	+	+
	1-60	+	+	+	+	+	0
	1-600	+	+	+	+	+	0
	1-6000	+	+	+	+	+	0
10	1-12	+	+	+	+	+	0
	1-60	0	0	0	0	0	0
	1-600	0	0	0	0	0	0
	1-6000	+	+	+	0	0	0
Normal defibrinated blood alone		+	+	+	+	+	+

0 = No growth.

+ = Growth.

\* Figures indicate the final dilution of serum.

an upper respiratory infection with rhinitis and pharyngitis during the 3-week period. The agglutinin titer in the serum of the first carrier was 1:32 and in the second, 1:128. No change in agglutinins was noted in the 2 samples of sera in either individual.

*Effect of sulfadiazine on the bactericidal test.*

In the preceding experiments, para-aminobenzoic acid was used in a concentration of 5 mgm. per cent. In the absence of this drug, small amounts of sulfadiazine exerted a misleading effect in bactericidal tests with the serum of patients. For example, a serum containing 8.0 mgm. per cent of sulfadiazine produced killing in a dilution of

1:120, but was entirely non-bactericidal when para-aminobenzoic acid was added.

*Comment.* The diminution in the bactericidal property of fresh undiluted serum during convalescence from Group I meningococcal infection may be analogous to the absence of bactericidal action in the undiluted serum of heavily immunized rabbits. In both instances this appears to be a manifestation of the prozone, since adequate dilution of the convalescent serum or of the immune rabbit serum results in bactericidal action when neutral complement is added. Similarly, inhibition of the bactericidal action of normal serum, which was observed with the convalescent sera of 4 patients, has also been shown to occur when immune rabbit serum is added to fresh normal rabbit or human serum. The mechanism of this action is not completely clear, but seems also to be related to the prozone phenomenon.

The strong bactericidal action in the fresh sera of 3 patients during the acute stage of the illness is difficult to explain. The same degree of bactericidal action is, however, encountered in many normal individuals and may represent the normal state in these 3 patients. If this is true, one might assume that this property does not constitute an adequate barrier against infection with the meningococcus, at least as far as the subarachnoid space is concerned. There are two alternative explanations which should also be considered: (1) The fresh serum of acutely ill, febrile patients has been shown by Tillett (24) to be bacteriolytic for hemolytic streptococci. It is possible that an analogous property may have caused the bacteriolysis of meningococci in the early serum of these 3 patients. Evidence is lacking to substantiate this at the present time. (2) It is conceivable that bactericidal antibody may have begun to develop very early in the course of the disease, perhaps even before the actual invasion of the meninges, and by the second or third day of florid symptoms, there may have been enough antibody to produce bacteriolysis in the fresh, undiluted serum. This is suggested, without direct supporting evidence, because it has been observed that after light immunization (*e.g.*, a single intravenous dose of living organisms), rabbits may develop bactericidal property in fresh, undiluted serum for resistant strains of meningococci. As has been mentioned, further im-

munization results in the disappearance of this property.

For practical purposes, the bactericidal test employing human defibrinated blood as complement with a resistant strain of meningococcus was found to be the most convenient method for estimating bactericidal antibody. The tests in which fresh normal serum was used as complement are of interest chiefly because of the differences which were brought out between meningococci of the same group. It was shown that if a strain was usually, but not always, susceptible to bacteriolysis in normal serum, it was also susceptible to bacteriolysis by immune serum when normal serum was used as complement. On the other hand, if a strain was generally resistant in fresh normal serum, it was also resistant when anti-serum and normal serum were combined, but was destroyed when defibrinated blood was used as complement. Except for those differences in susceptibility, the strains were indistinguishable in their general characteristics, including morphology, agglutinability, quellung, and virulence for mice (17).

The presence of bactericidal antibody in the convalescent serum was in each instance associated with specific agglutinins and complement-fixing antibodies. The number of tests performed was not sufficient to determine a quantitative relation between the results. The bactericidal test appears, as might be expected, to be a more highly sensitive method than the agglutination or complement-fixation tests. The time when bactericidal antibody begins to develop has not been shown in this study. It has been shown, however, that antibody of high titer is present between the seventh and fourteenth days of the disease.

The appearance of bactericidal antibody in the serum of a Group I carrier, 3 weeks after a previous negative test, is of some interest. Unfortunately, the duration of the carrier state in this individual is not known. It is possible that it may have begun shortly before the first test, and the positive result in the second test may have represented a delayed response to infection. If, however, the carrier state had been of longer duration (which the agglutinin titer of 1:128 in the first serum might suggest), it is also possible that the appearance of bactericidal antibody may have been the result of a transient reinvasion during

an upper respiratory infection, unaccompanied by the usual symptoms of meningococcal infection.

#### DISCUSSION

The appearance of specific antibody during convalescence from meningococcal meningitis has been demonstrated by all except two of the methods employed. Negative results were obtained in the plate precipitation and mouse-protection tests. In the first, this may have been due to insufficient concentrations of serum. In the mouse-protection test, some of the positive results may actually have been due to the presence of antibody, but the potential interference by small amounts of sulfadiazine in the sera renders the test inconclusive.

The most consistently positive results were obtained by the agglutination test, in which 35 of 36 convalescent sera were positive. Agglutinins were also encountered, however, during the early stage of the disease in some patients, as well as in the sera of Group I carriers and contacts. The significance of a single observation during the course of the disease is therefore doubtful, and the chief value of the test lies in the demonstration of an actual increase in titer between the acute and convalescent stages. The complement-fixation test yielded fewer positive results in the convalescent sera, but, with the exception of 1 Group I carrier, it was only positive within this group. The quellung test, by the incubation method, was positive in only 6 of 25 convalescent sera, and probably indicates a high level of antibody. The bactericidal test, although beset with technical difficulties, seemed to be the most sensitive method for demonstrating antibody and provided the most striking differences between acute and convalescent sera.

No consistent correlation could be seen between the degree of antibody response and the severity or duration of illness in this series. Patients with relatively mild clinical courses and prompt recovery could not be distinguished from more severely ill cases on the basis of these tests. It should be mentioned, however, that the general course in the majority of cases in this series was approximately the same, and the number of patients at either extreme was relatively small. The response to sulfonamide treatment was rapid in

most cases, and only 3 cases of resistant infection were observed. One of these patients, who eventually recovered, developed a high level of antibody, as measured by agglutination, complement-fixation, and bactericidal tests. The second, an infant aged 2 years, had no detectable antibody by any of the tests after 3 weeks and died shortly thereafter. A third patient, who received therapeutic antiserum, died after 3 days; the serum on the day of death possessed agglutinins in a titer of 1:64 and complement-fixing antibody in a titer of 1:32. The development of specific antibody was in no way related to the virulence of the infecting organisms for mice, nor to their ability to survive in fresh normal human serum.

The results of tests with carrier sera seem to cast some light on the problem of the carrier state. In the preceding paper (17), it was shown that the Group I carrier strains were of the same order of virulence for mice as the Group I strains from the cerebrospinal fluid of cases. In the present study, 4 Group I carriers were found to possess antibody levels which were as high as or higher than those in convalescent patients. Agglutination tests were positive in all, mouse-protection tests in 2, and the bactericidal test and complement-fixation test each in 1. These observations indicate that the Group I carrier state is, as Rake (11) has suggested, an actual infection which may remain limited to the nasopharynx. On the other hand, the absence of detectable antibody in Group II carriers, whose organisms were avirulent, indicates that such organisms may be passive saprophytes without potential invasiveness. This probably does not apply to Group II carrier strains during outbreaks of Group II cases, since Silverthorne and his co-workers (10) have shown that these organisms may be virulent.

The results of the agglutination tests with contact sera have implied that there is some relation between the repeated exposure to meningococci and the development of specific antibody. It has not been shown, however, that this process prevents the development of meningitis.

#### SUMMARY

1. Several methods have been employed for the demonstration of specific antibody in the sera of 36 patients with meningococcal meningitis during the acute and convalescent stages of the disease,



and in the sera of 4 Group I carriers, 6 Group II carriers, and 59 Group I contacts.

2. The *agglutination test* was positive in 33 of 34 convalescent sera from Group I cases and in 2 convalescent sera from Group II cases. Agglutinins were also present in 12 sera in the acute stage, but a definite rise in titer between the acute and convalescent sera was found in all except 2 patients. Agglutinins were shown to appear as early as the seventh day and to persist as long as the twenty-eighth day. The sera of 4 carriers of Group I organisms had agglutinin titers of between 1:32 and 1:128. The sera of 5 of 6 carriers of Group II organisms had no agglutinins for homologous or Group I strains. The serum of 1 Group II carrier had agglutinins for both strains in a titer of 1:16. Of 59 normal Group I contacts, 40 had titers of 1:8 or lower; 11 of the 19 contacts with higher titers were found to be concentrated in two households.

3. The *mouse-protection test* was found to be impractical for use with sera from patients who had received sulfonamide therapy, because of the protective action of small amounts of drug and the difficulty in inhibiting this action with para-aminobenzoic acid. Of 2 Group II carrier sera, 1 protected against 100,000 and the other against 10 50 per cent lethal doses. Of 14 Group I contact sera, 1 protected against 100,000 and 1 against 10 50 per cent lethal doses.

4. *Quellung* was produced by 6 convalescent sera from Group I cases when the organisms were incubated in the serum for from 2 to 4 hours.

5. The *plate precipitation test* was negative with 4 convalescent sera which possessed Group I antibody by other methods.

6. The *complement-fixation test* was positive in 16 of 26 convalescent sera from Group I cases. Fourteen sera were negative during the acute stage of the disease. One of 3 Group I carrier sera was positive. Thirty sera from Group I contacts were negative.

7. *Bactericidal tests* with the fresh, undiluted sera of 3 Group I patients against their own strains showed a diminution in bactericidal property during convalescence. The bactericidal action of fresh normal serum for a susceptible strain of Group I meningococcus was inhibited by the addition of convalescent sera from 4 patients, while the acute sera of these patients was without effect. This

effect was apparently due to a prozone, as will be discussed in a later paper on the mechanism of bactericidal action against the meningococcus (23). With neutral fresh human serum or defibrinated blood as complement, strong bactericidal action was demonstrable with the convalescent sera of 4 patients in dilutions ranging from 1:600 to 1:6,000. In simultaneous tests, no bactericidal action was exerted by serum in the acute stage of the disease. One Group I carrier developed bactericidal action in a serum dilution of 1:60, during a 3-week period of observation.

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# INVESTIGATIONS OF MENINGOCOCCAL INFECTION. III. THE BACTERICIDAL ACTION OF NORMAL AND IMMUNE SERA FOR THE MENINGOCOCCUS<sup>1</sup>

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The immunological mechanisms which enable the infected host to kill the meningococcus have constituted an unsettled problem since the earliest studies of this organism. It has usually been assumed that bacteriolysis plays an important role, especially since normal sera possess bactericidal properties for many strains of meningococci, but many investigators have encountered difficulties in the demonstration of bactericidal antibodies in immune sera. For example, Murray (1) stated that immune horse sera failed to exhibit bacteriolytic antibodies, and suggested that "the precipitin reaction interfered with the result by fixing the available alexine." Gordon (2) found antimeningococcal sera "remarkably deficient in bacteriolysin." Matsunami and Kolmer (3) reported that immune sera possessed bactericidal properties of low order, and found that higher dilutions of serum were sometimes more bactericidal than lower ones. Silverthorne and his associates (4), in recent years, have applied the bactericidal test to the study of patients and immunized animals, employing fresh citrated blood for most of the tests, and have reported consistent increases in the bactericidal power of blood from convalescent patients as well as from immunized animals; these reports do not, however, deal with actual titrations of immune serum in the presence of neutral complement.

Because of the frequent finding of strong bactericidal activity in fresh, undiluted normal serum,<sup>3</sup> the bactericidal test assumes greater significance as a quantitative measure of antibody when bactericidal property can be demonstrated in considerable dilutions of antiserum. The present com-

munication is concerned with the conditions under which such a test is possible, and also with the effect of excessive amounts of specific antibody upon the bactericidal mechanism *in vitro* and *in vivo*. The application of the bactericidal test to the study of antibody formation in human beings has been described in the preceding paper (5).

## MATERIAL AND METHODS

**Bactericidal test.** The method was similar to that employed in a previous study of the influenza bacillus (6). The various strains of meningococci used in the experiments were maintained in storage on carbon-dioxide ice, each strain being suspended in milk and distributed in a large number of sealed pyrex tubes. During each week, the strains were maintained by daily transfers on blood agar or serum-dextrose agar slants; at the end of the week, these strains were discarded and a new lot removed from storage. For each test, the organisms were grown for 6 hours on serum-dextrose agar slants, or, in some of the experiments, on casein-hydrolysate starch-agar medium. Suspensions of this growth were made in broth, and 10-fold serial dilutions were prepared in broth. The turbidity of the original suspension was adjusted by means of a photoelectric turbidometer so that 0.1 ml. of the 10<sup>-4</sup> dilution contained approximately 100 organisms. Plate counts were also carried out, using the 10<sup>-5</sup> and 10<sup>-6</sup> dilutions, as an additional check on the number of organisms.

The materials used in the tests were mixed in the following proportions: complement, 0.25 ml.; heat-inactivated antiserum, 0.05 ml.; dilutions of organisms from 10<sup>-4</sup> to 10<sup>-6</sup>, 0.05 ml. In tests of the bactericidal action of fresh serum or defibrinated blood, volumes of 0.25 ml. were mixed with 0.05 ml. of organisms. The mixtures were made in small pyrex tubes which were then sealed and placed in an automatic rotator in an incubator. Twenty hours was adopted as the time of incubation in all the tests, after which the tubes were opened, the contents thoroughly mixed, and approximately 0.05 ml. from each tube planted on the surface of a blood agar plate. The presence of any growth on the plate was taken to indicate the absence of bactericidal property in the serum tested for the number of organisms inoculated.

**Complement.** Fresh normal serum or defibrinated blood was obtained from healthy adults with no history of pre-

<sup>1</sup> This study was aided in part by a grant from the William W. Wellington Memorial Research Fund.

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<sup>3</sup> Hereinafter the term "fresh serum" will indicate fresh undiluted serum.

vious meningococcal infection or exposure. Fresh rabbit, guinea pig, and horse sera were also employed as complement. The sera were always used within a few hours after bleeding, and were kept on ice during the interim.

*Antisera.* The following antisera were used in the experiments: (1) several samples of commercial Group I rabbit antiserum, refined and concentrated,<sup>4</sup> (2) Group I horse antiserum, unconcentrated,<sup>5</sup> (3) convalescent serum from a number of patients with Group I meningococcal meningitis, and (4) several lots of serum prepared in this laboratory by the immunization of rabbits and guinea pigs with single strains of Group I meningococci, recently isolated from the cerebrospinal fluid of patients. The method of immunization consisted of daily injections, intravenously in rabbits and intraperitoneally in guinea pigs, of 1.0 ml. of a turbid saline suspension of living organisms, in 5-day courses, with rest intervals of 4 to 5 days between each course. The animals were bled 1 week or more after the last immunizing dose. The antisera were inactivated by heating at 56° C. for 30 minutes.

*Titration of hemolytic complement.* These were performed in some of the experiments. The following method was used: Varying quantities of the serum to be tested were diluted in normal saline so as to make a final volume of 0.5 ml. To this were added 2 units of amboceptor contained in 0.5 ml. of saline, and 0.5 ml. of a 2 per cent suspension of washed sheep red corpuscles. The mixtures were incubated at 37.5° C. for 30 minutes. The last tube in which complete hemolysis occurred was selected as the endpoint. For convenience, the titer of complement has been expressed in units which represent the reciprocal of the final dilution of fresh serum.

## RESULTS

### I. The bactericidal action of normal and immune sera

#### *The bactericidal property of normal serum for meningococci*

Different strains of meningococci exhibited wide differences in their susceptibility to the bactericidal action of the same sample of fresh normal human serum. These differences could not be correlated with the virulence of the organisms for mice, nor with the serological grouping of the strains. A detailed account of this observation has been presented in the first paper of this series (7).

Some variation was also encountered in the bactericidal potency of fresh serum from different normal individuals. This variation was not, however, as striking as the differences in susceptibility between the strains themselves. The sera of 4 in-

fants between the ages of 6 months and 1 year were somewhat less bactericidal than the sera of adults, but even these infant sera possessed strong bactericidal action for 1 virulent strain of Group I meningococcus (Strain No. 30). Fresh serum from normal rabbits had approximately the same degree of bactericidal action for meningococci as adult human serum. Fresh guinea pig serum, as previously shown by Silverthorne (8), produced bacteriolysis of Group I meningococci with regularity, but not of Group II organisms.

The bactericidal action of normal serum appeared to be dependent upon the presence of complement, since it disappeared in every instance when the serum was heated at 56° C. for 30 minutes. This was demonstrated with 3 different strains, all of which were susceptible to the bactericidal action of fresh human, rabbit, and guinea pig serum.

In general, it was possible to divide Group I meningococci into two main classes, based upon the bactericidal action of normal human and rabbit serum, namely, resistant and susceptible strains. The line of division between these two groups was not sharply definable, since occasional normal sera were encountered which failed to kill the generally susceptible strains, while others

TABLE I

*Comparison of the bactericidal action of fresh serum and defibrinated blood of 7 normal individuals with the resistant strain of Group I meningococcus (No. 21)*

Person	Test	Dilution of organisms *					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
T.	Serum Blood	+	+	+	+	+	+
G.	Serum Blood	+	+	+	+	+	+
B.	Serum Blood	+	+	+	+	0	0
Ms.	Serum Blood	+	+	+	+	+	+
Ro.	Serum Blood	+	+	+	+	+	+
Hs.	Serum Blood	+	+	+	+	+	+
St.	Serum Blood	+	+	+	+	+	+

\* 0.05 ml. of 10<sup>-6</sup> dilution = 50 organisms.

+ = Growth.

0 = No growth.

<sup>4</sup> Supplied by The Lederle Laboratories, Inc.

<sup>5</sup> Supplied by Dr. Leo Rane, of the Massachusetts State Antitoxin Laboratory.

caused bacteriolysis of the generally resistant strains. Despite this flexibility, however, this rough classification was found to be useful, since the two groups presented important differences in the bactericidal tests with immune sera.

Two representative Group I strains were selected for the experiments to follow, designated as strain No. 2 (usually susceptible) and strain No. 21 (usually resistant).

#### *The bactericidal property of normal defibrinated blood*

In some normal individuals, the bactericidal action of defibrinated blood was the same as that of fresh serum. In others, strong bactericidal activity for the resistant strain was present in defibrinated blood and absent in serum. These differences between individuals are illustrated in Table I.

#### *The effect of immune serum in the bactericidal test with the resistant strain (No. 21)*

The bactericidal action of heat-inactivated rabbit antiserum for the resistant strain was tested in the presence of human complement, rabbit com-

plement, and normal human defibrinated blood. The following results were obtained:

(1) With rabbit serum as complement, strong bactericidal action was produced by rabbit antiserum in dilutions as high as 1:60,000. A conspicuous prozone was present (Table II).

(2) With human serum as complement, no bactericidal action was demonstrable in any dilution of rabbit antiserum (Table II).

(3) With normal human defibrinated blood as complement, however, bactericidal action was produced by high dilutions of rabbit antiserum, again with a prozone (Table II).

(4) Bactericidal antibody could be demonstrated in human convalescent serum in the presence of human defibrinated blood, but not when

TABLE III

*Bactericidal tests with the susceptible (No. 2) and resistant (No. 21) strains of Group I meningococcus with human complement lacking bactericidal action for strain No. 2*

Strain	Dilution of rabbit antiserum	Dilution of organisms *					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
No. 2	1-60	+	+	+	+	+	+
	1-600	+	+	0	0	0	0
	1-6000	+	+	0	0	0	0
	1-60,000	+	+	+	0	0	0
	None	+	+	+	+	+	+
No. 21	1-60	+	+	+	+	+	+
	1-600	+	+	+	+	+	+
	1-6000	+	+	+	+	+	+
	1-60,000	+	+	+	+	+	+
	None	+	+	+	+	+	+

\* 0.05 ml. of 10<sup>-6</sup> dilution = 50 organisms.

+ = Growth.

0 = No growth.

human serum was employed as complement. These results have been presented in detail in the preceding paper (5).

#### *The effect of immune serum in the bactericidal test with the susceptible strain (No. 2)*

It has already been mentioned that an occasional normal human serum was encountered which lacked bactericidal action against the susceptible strain. When the fresh serum of such an individual was used as complement, strong bactericidal action was demonstrated in dilutions of rabbit antiserum. In a simultaneous test, the same complement was ineffective against the resistant strain (Table III).

TABLE II

*Bactericidal tests with the resistant strain (No. 21), using rabbit antiserum in presence of different types of complement*

Complement	Antiserum dilution †	Dilution of organisms *					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
Rabbit serum diluted 1-2	1-60	+	+	+	+	+	+
	1-600	+	+	+	+	+	0
	1-6000	+	+	0	0	0	0
	1-30,000	+	+	+	0	0	0
	1-60,000	+	+	+	0	0	0
	None	+	+	+	+	+	+
Human serum diluted 1-2	1-6	+	+	+	+	+	+
	1-60	+	+	+	+	+	+
	1-600	+	+	+	+	+	+
	1-6000	+	+	+	+	+	+
	1-60,000	+	+	+	+	+	+
	1-300,000	+	+	+	+	+	+
Human defibrinated blood	1-600	+	+	+	+	+	+
	1-3000	+	0	0	0	0	0
	1-6000	+	0	0	0	0	0
	1-30,000	+	0	0	0	0	0
	1-60,000	+	+	+	0	0	0
	None	+	+	+	+	+	0

\* 0.05 ml. of 10<sup>-6</sup> dilution = 50 organisms.

+ = Growth.

0 = No growth.

† Expressed as final dilution.

Bactericidal antibody was also demonstrated in human convalescent serum by this method (5).

#### *The bactericidal action of fresh immune serum*

The fresh sera of a number of rabbits were tested for bactericidal action with the resistant (No. 21) and susceptible (No. 2) strains, before and after active immunization with Group I meningococci. A similar experiment was performed with a guinea pig. The results were as follows:

(1) Light immunization against the resistant strain resulted in the development of good bactericidal action for this organism in the fresh serum of 2 rabbits. One animal received a single intravenous injection of organisms and was bled 9 days later (Rabbit 39). The second rabbit was given 7 injections and was bled one week after the last dose (Rabbit 12) (Table IV).

TABLE IV

*Bactericidal action of fresh immune rabbit sera for the resistant strain (No. 21) following immunization with this strain*

Rabbit number*	Number of injections of antigen	Dilution of organisms †					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
39	1	+	+	0	0	0	0
12	7	+	+	0	0	0	0
7	19	+	+	+	+	+	+
3	32	+	+	+	+	+	+
Normal	0	+	+	+	+	+	+

\* Sera diluted 1-2.

† 0.05 ml. of 10<sup>-6</sup> dilution = 50 organisms.

+ = Growth. 0 = No growth.

(2) Heavy immunization against the resistant strain, consisting of 19 injections in one rabbit (Rabbit 7) and 32 injections in another (Rabbit 3), resulted in no demonstrable bactericidal action in the fresh serum (Table IV).

(3) Heavy immunization against the susceptible strain, amounting to at least 20 injections of organisms, resulted in the loss of bactericidal action in the fresh serum of 3 rabbits (Table V). Furthermore, bactericidal action for this strain was also lacking in the fresh sera of the 2 rabbits which were heavily immunized against the resistant strain.

(4) The sera of these 5 heavily immunized rabbits, although devoid of bactericidal action when undiluted or diluted 1:2, contained abundant bac-

TABLE V

*The loss of bactericidal action in fresh serum for the susceptible strain (No. 2) following prolonged immunization*

Animal	Number of injections of antigen	Dilution of organisms *					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
Rabbit	0	+	+	0	0	0	0
	30	+	+	+	+	+	+
Rabbit	0	+	+	+	0	0	0
	12	+	+	+	+	0	0
	32	+	+	+	+	+	+
Rabbit	0	+	+	+	0	0	0
	12	+	+	+	0	0	0
	32	+	+	+	+	+	+
Guinea pig	0	0	0	0	0	0	0
	32	+	+	+	+	+	0

\* 0.05 ml. of 10<sup>-6</sup> dilution = 50 organisms.

+ = Growth.

0 = No growth.

tericidal antibody. This could be demonstrated by inactivating and diluting each serum in the presence of normal rabbit complement, and testing with the resistant strain. In each instance, a prozone was present with dilutions as high as 1:60, but strong bactericidal action was exerted by dilutions of 1:600 and 1:6,000. An illustrative titration is shown in Table VI.

No bactericidal action was present in any dilution of the fresh immune sera in normal saline, nor in normal rabbit serum which had been inactivated. The reason for this may be that dilutions of fresh serum which were within the range of complement activity were also within the range of the prozone. It may be added that titrations

TABLE VI

*The bactericidal action of fresh immune rabbit serum for the resistant strain (No. 21) compared with dilutions of the same serum in the presence of normal rabbit complement*

Test	Dilution of antiserum	Dilution of organisms *					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
Fresh anti-serum	Undiluted	+	+	+	+	+	+
Normal rabbit complement diluted 1-2	1-60	+	+	+	+	+	+
	1-600	+	+	0	0	0	0
	1-3000	+	+	+	0	0	0
	1-6000	+	+	+	0	0	0
Normal rabbit complement alone		+	+	+	+	+	+

\* 0.05 ml. of 10<sup>-4</sup> dilution = 50 organisms.

+ = Growth.

0 = No growth.

of hemolytic complement in these immune sera revealed no difference from the complement titers of normal rabbit sera.

(5) A guinea pig, which was immunized with the resistant strain for a total of 32 injections, showed an almost complete loss in the bactericidal action of his fresh serum for this strain (Table V). As in the case of the immune rabbit sera, the addition of 1:6,000 dilutions of this animal's immune serum, inactivated, to normal rabbit complement, resulted in strong bactericidal action.

The fresh serum and defibrinated blood of 2 horses, which had undergone immunization with living Group I meningococci during the preceding year, were tested for bactericidal action against the resistant and susceptible strains. The organisms survived in dilutions of  $10^{-4}$  in the serum and blood of both horses. When the sera were inactivated and tested in the presence of fresh normal horse serum with the resistant strain, no bactericidal action occurred. However, when normal horse defibrinated blood was employed instead of serum as complement, killing of this strain took place with antiserum in dilutions of 1:60,000. A prozone was observed.

During the course of immunization, one of the immunized horses developed bacteremia, due to a

strain of Group I meningococcus. The fresh serum of this animal was completely lacking in bactericidal property for this strain.

Observations which are considered to be analogous to these results with animal sera were made in 3 patients during the course of Group I meningococcal infection (5). The fresh serum of each patient was tested for bactericidal action against his own strain of meningococcus during the acute and convalescent stages of the disease. Each patient was found to have good bactericidal action during the acute stage, which disappeared almost completely during convalescence. At the latter time, each of the patients had developed specific agglutinins and complement-fixing antibodies. Furthermore, bactericidal antibodies were demonstrable when the convalescent sera were diluted and added to normal human defibrinated blood.

## II. The inhibitory effect of immune sera upon the bactericidal action of normal sera, for Group I meningococci

The foregoing experiments have indicated that antisera for Group I meningococci contain demonstrable bactericidal antibodies, but these are readily masked by a prozone which may occur in the fresh undiluted antiserum as well as in dilutions of inactivated antiserum plus normal complement. The loss of natural bactericidal action in fresh serum, following immunization, suggested that this prozone was due to actual inhibition by antibody excess rather than simply the passive failure of bactericidal antibody to function. It was then of interest to determine whether similar inhibition could be induced by the addition of immune serum to fresh normal serum.

When rabbit antiserum, inactivated by heating, was added to fresh normal rabbit serum, the bactericidal action of the latter for the susceptible strain disappeared. The degree of inhibitory effect appeared to be related to the antibody titer of the antiserum, as is shown in Table VII. Antiserum A, with a complement fixation titer of 1:64, caused complete inhibition when diluted 1:24 and incomplete inhibition in higher dilutions as far as 1:384. Antiserum B, with a complement fixation titer of 1:2048, produced complete inhibition in a dilution of 1:384, and incomplete inhibition in dilutions as high as 1:1536.

TABLE VII

Comparison of the inhibitory effect of 2 rabbit antisera upon the bactericidal action of normal rabbit serum

Immune serum	Dilution	Dilution of organisms *					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
Rabbit A	1-12	+	+	+	+	+	+
	1-24	+	+	+	+	+	+
	1-48	+	+	+	+	+	0
	1-96	+	+	+	+	0	0
	1-192	+	+	0	+	0	0
	1-384	+	+	0	+	0	0
	1-768	+	+	0	0	0	0
	1-1536	+	+	0	0	0	0
Rabbit B	1-12	+	+	+	+	+	+
	1-24	+	+	+	+	+	+
	1-48	+	+	+	+	+	+
	1-96	+	+	+	+	+	+
	1-192	+	+	+	+	+	+
	1-384	+	+	+	+	+	+
	1-768	+	+	0	+	0	0
	1-1536	+	+	+	0	0	0
Control—Complement alone		+	+	0	0	0	0

\* 0.05 ml. of  $10^{-6}$  dilution = 50 organisms.

+ = Growth. 0 = No growth.



A similar inhibitory effect was exerted by rabbit antiserum upon the natural bactericidal action of fresh normal human and guinea pig sera. Several samples of horse antiserum also produced inhibition. Convalescent sera from 4 patients with Group I meningococcal meningitis caused inhibition of the bactericidal action of normal human serum, as was shown in the preceding paper (5). No inhibition of natural bactericidal action was observed with numerous normal serum controls, nor with horse antisera for pneumococcus Type II, Friedländer's bacillus, and *H. influenzae*, nor with the convalescent serum of 6 patients with pneumococcal pneumonia.

TABLE VIII

*The inhibitory effect of rabbit antiserum in varying dilutions of fresh normal serum*

Complement dilution	Antiserum dilution	Dilution of organisms *					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
Undiluted	1-6	+	+	0	0	0	0
	1-60	0	0	0	0	0	0
	1-600	0	0	0	0	0	0
	None	0	0	0	0	0	0
1-2	1-6	+	+	+	+	+	+
	1-60	+	0	0	0	0	0
	1-600	+	+	0	0	0	0
	None	+	+	0	0	0	0
1-4	1-6	+	+	+	+	+	+
	1-60	+	+	0	0	0	0
	1-600	+	+	0	0	0	0
	None	+	+	+	+	0	0

\* 0.05 ml. of 10<sup>-6</sup> dilution = 50 organisms.

+ = Growth. 0 = No growth.

No inhibitory effect of antiserum could be demonstrated in some samples of fresh normal serum which were possessed of a very high degree of natural bactericidal action. It was found that the inhibition was more striking with 1:2 dilutions of normal serum than when the latter was used undiluted. In higher dilutions of normal serum, the natural bactericidal property diminished rather abruptly, and the addition of dilutions of antiserum produced some enhancement of bactericidal action while the zone of inhibition became recognizable as a prozone (Table VIII).

The rabbit antisera used in the foregoing tests had no anti-complementary effect upon fresh normal rabbit or guinea pig serum. An experiment was conducted to determine whether the in-

hibitory effect of antiserum was caused by fixation of complement in the normal serum through the interaction of specific antibody with antigen. Tubes containing fresh normal rabbit serum, and fresh normal serum plus inactivated rabbit antiserum, were inoculated with the 10<sup>-5</sup> broth dilution of the susceptible strain. The tubes were then incubated in a candle-jar for 2 hours, after which plate counts were made with 0.1 ml. from each tube. Titrations of hemolytic complement were performed before and after incubation. It was found that complete killing of the organisms had taken place in the fresh normal serum, while the plate count from the tube containing normal serum plus antiserum showed 260 colonies in 0.05 ml. The titer of hemolytic complement remained approximately the same in both tubes, indicating that the survival of organisms in the presence of specific antiserum was not caused by the fixation of complement.

The inhibitory effect of rabbit antiserum was sharply reduced by absorption with Group I organisms. This was shown with 3 different samples of antiserum, which were inactivated by heating and mixed with an equal volume of a turbid suspension of washed meningococci. The mixtures were incubated for 2 hours at 37.5° C. and kept in an icebox for 18 hours. Three samples of normal rabbit serum were similarly treated. Following absorption, each serum was filtered through a Seitz pad and tested for its effect upon the bactericidal action of fresh normal rabbit serum. Simultaneous tests were made with 1:2 dilutions of the original, unabsorbed

TABLE IX

*The reduction in the inhibitory action of immune rabbit serum following absorption with Group I meningococci*

Complement	Rabbit serum	Dilution of organisms *					
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
Fresh, normal rabbit serum, diluted 1-2	Normal before absorption	0	0	0	0	0	0
	Normal after absorption	0	0	0	0	0	0
	Immune before absorption	+	+	+	+	+	+
	Immune after absorption	+	+	+	0	0	0
	Complement alone	+	0	0	0	0	0

\* 0.05 ml. of 10<sup>-6</sup> dilution = 50 organisms.

+ = Growth. 0 = No growth.

sera. The results are shown in Table IX. The normal rabbit serum produced no effect on the bactericidal action of fresh normal serum, either before or after absorption. The immune serum, before absorption, caused inhibition of bactericidal action for all dilutions of organisms, while following absorption, the inhibition extended only as far as the  $10^{-3}$  dilution.

### III. The effect of intravenous injections of anti-serum upon the bactericidal action of fresh serum

#### Method

Normal rabbits, weighing between 2000 and 3000 grams, were injected intravenously with varying amounts of Group I antimeningococcus serum. Three varieties of antiserum were employed: (1) horse antiserum, unconcentrated; (2) commercial rabbit antiserum, refined and concentrated; and (3) rabbit antiserum prepared in this laboratory. In addition, some rabbits were injected with a preparation of concentrated normal human globulin. The animals were bled immediately before each injection and again after 30 minutes. Serum from each bleeding was obtained by centrifugation as soon as clotting occurred, and was kept on ice. Bactericidal tests and complement titrations were performed simultaneously, within a few hours of the time of bleeding. The sera were diluted 1:2 in saline for all the bactericidal tests.

Two normal human adults were injected intravenously with commercial rabbit antiserum, refined and concentrated, in 25 ml. and 40 ml. quantities. Bactericidal tests with defibrinated blood as well as serum, and complement titrations, were performed before and 30 minutes after the injection of antiserum.

#### Results

The results of the experiments with rabbits are summarized in Table X.

The injection of small amounts of horse antiserum (0.1 ml. and 0.5 ml.) was followed by the appearance of strong bactericidal action in the fresh serum for the resistant strain (No. 21). When larger doses were injected, this effect was no longer seen, and when 10 ml. were injected, the bactericidal property for the susceptible strain

(No. 2) disappeared (Experiments 1 to 5). When a single rabbit was given successive injections of increasing amounts of antiserum at half hourly intervals, bactericidal action appeared after the 0.5 ml. dose, diminished after 1.0 ml., and disappeared after 2.5 ml. (Experiment 5). No diminution in hemolytic complement occurred following any dose of horse antiserum.

The concentrated rabbit antiserum was found to be highly anticomplementary when injected intravenously into rabbits. Following doses of 0.5 ml. or greater, no hemolytic complement could be detected by the method employed. In some animals, this effect persisted for as long as 24 hours after injection. This antiserum was also found to be anticomplementary *in vitro*, in dilutions as high as 1:1024. The effect on the bactericidal action of fresh serum was similar to that of horse antiserum, *i.e.*, small amounts of antiserum produced enhancement of bactericidal property for the resistant strain, but doses of 2.5 ml. or greater caused the loss of bactericidal action for the susceptible strain (No. 2) (Experiments 6 to 12). It should be noted that an increase in killing occurred, despite an accompanying diminution in titratable complement, with doses of 0.5 ml. and 1.0 ml.

The effect of an anticomplementary substance lacking specific antibody was determined by injecting varying amounts of concentrated human globulin, which had approximately the same *in vitro* anticomplementary effect as the concentrated rabbit antiserum. The injection of 1.0 ml. resulted in the disappearance of detectable complement for 18 hours and also caused the loss of bactericidal action for the susceptible strain (No. 2). The same effect was produced by an injection of 5.0 ml., while doses of less than 1.0 ml. had no significant effect upon either complement or bactericidal property (Experiments 13 to 16).

Several experiments were performed with rabbit antiserum prepared in this laboratory, which, like the horse antiserum, had no anticomplementary action. Definite results were not obtained, and unfortunately the experiments could not be extended because of an inadequate supply of this serum. No effect upon bactericidal action was produced by the injections except that in 2 animals there was some diminution following injections of 5 ml. and 10 ml. amounts (Experiments

TABLE X  
*The bactericidal action and complement titer of rabbit serum before and after  
the injection of antiserum and normal human globulin*

Material injected	Experiment number	Strain	Dose of antiserum (ml.)	Time	Dilution of organisms						Complement (units)*
					10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	
Horse antiserum	1	21	0.1	Before After	+	+	+	+	+	+	15 15
	2	21	0.5	Before After	+	+	+	+	+	+	25 25
	3	21	1.0	Before After	+	+	+	+	+	+	25 25
	4	2	10.0	Before After	+	+	+	0	0	0	15 15
	5	21	None	Before	+	+	+	+	+	+	5
			0.1	After	+	+	0	0	0	0	5
			0.5	After	+	0	0	0	0	0	5
			1.0	After	+	+	+	+	0	0	5
			2.5	After	+	+	+	+	+	+	5
			5.0	After	+	+	+	+	+	+	5
Concentrated rabbit antiserum	6	21	0.1	Before After	+	+	+	+	+	+	25 25
	7	21	0.5	Before After	+	+	+	+	+	+	5 <3
	8	21	1.0	Before After	+	+	+	+	+	0	15 <3
	9	21	2.5	Before After	+	+	+	+	+	+	7.5 <3
	10	21	5.0	Before After	+	+	+	+	+	+	5 <3
	11	2	2.5	Before After	+	+	+	0	0	0	7.5 <3
	12	2	5.0	Before After	+	+	0	0	0	0	5 <3
Concentrated rabbit antiserum which were natural bactericidal, and inhibition was normal serum diluted. In high natural bactericidal action abruptly, and the serum produced secondary action while the zone of inhibition was not pronounced	13	2	0.1	Before After	+	+	0	0	0	0	15 15
	14	2	0.5	Before After	+	+	0	0	0	0	7.5 5
	15	2	1.0	Before After	+	+	+	0	0	0	7.5 <3
	16	2	5.0	Before After	+	+	0	0	0	0	7.5 <3
	17	21	5.0	Before After	+	+	+	+	0	0	15 15
	18	21	10.0	Before After	+	+	+	+	+	+	15 15
	19	21	20.0	Before After	+	+	+	+	+	+	25 25
The rabbit antiserum had no anti-complement activity. The normal rabbit or guinea pig serum complement was conducted to		2	5.0	Before After	+	+	+	0	0	0	15 15
		2	10.0	Before After	+	0	0	0	0	0	15 15

\* It has been expressed in units which represent the reciprocal of the highest final dilution giving complete hemolysis.

17 and 21). The agglutinin titer of this serum was 1:640.

The results of the injection of 25 cc. of concentrated rabbit antiserum in a normal man are shown in Table XI. No effect on hemolytic complement was observed following the injection, although this was the same lot of serum which had previously been shown to be highly anticomplementary in rabbits. The bactericidal property of fresh serum and defibrinated blood for the suscep-

TABLE XI

*The bactericidal action of human serum and defibrinated blood for the susceptible (No. 2) and resistant (No. 21) strains of Group I meningococci, before and after the injection of 25 ml. of concentrated rabbit antiserum*

Strain	Material tested	Time	Dilution of organisms *					
			10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
2	Serum 1-2	Before	0	0	0	0	0	0
		After	+	+	+	+	0	0
	Defibrinated blood	Before	0	0	0	0	0	0
		After	+	+	0	0	0	0
21	Serum 1-2	Before	+	+	+	+	+	+
		After	+	+	+	+	+	+
	Defibrinated blood	Before	+	+	+	+	+	0
		After	+	+	0	0	0	0

\* 0.05 ml. of 10<sup>-6</sup> dilution = 50 organisms.

+ = Growth. 0 = No growth.

tible strain (No. 2) was definitely diminished 30 minutes after the injection. At the same time, however, a notable increase occurred in the bactericidal action of defibrinated blood for the resistant strain (No. 21). No killing of the latter strain occurred in the serum alone. Comparable results were obtained when a second individual was injected with 40 cc. of concentrated rabbit antiserum; the titer of hemolytic complement also remained the same in this subject.

#### IV. *The effect of active and passive immunization upon the duration of induced bacteremia in rabbits*

##### *Active immunization*

Three rabbits which had been actively immunized against Group I meningococci by 32 injections of living organisms, and 3 normal control rabbits of the same weights, were injected intravenously with 1.0 ml. of a freshly prepared turbid

suspension, in saline solution, of the resistant strain (No. 21). Blood cultures were made by allowing approximately 0.5 ml. of blood to drop from an incised ear vein into 5.0 ml. of broth. Cultures were made at 30 minutes, 1 hour, 4 hours, 8 hours, 24 hours, and 48 hours after the injection of organisms.

A considerable variation in resistance to bacteremia was encountered in both groups of animals, but the immunized rabbits did not appear to be significantly different from the normal controls. In the 3 immune rabbits, the last positive cultures were obtained at 4 hours in 2, and at 8 hours in the third. In the control rabbits, the last positive cultures were obtained at 1 hour, 8 hours, and 24 hours.

##### *Passive immunization*

Three rabbits were injected intravenously with 5.0 ml. of concentrated rabbit antiserum, known to be anti-complementary; 30 minutes later, each rabbit received 1.0 ml. of a freshly prepared turbid suspension, in saline, of the susceptible strain (No. 2). At the same time, 3 control rabbits were given the same dose of organisms. Blood cultures were made at intervals, as in the preceding experiment.

Meningococci were not cultured from the blood of any of the control animals. One of the rabbits which received antiserum had a positive culture at 8 hours, the second rabbit at 24 hours, and the third at 48 hours.

In a similar fashion, 2 rabbits were given anti-meningococcal horse serum (Massachusetts No. 758), intravenously, in doses of 5 ml. and 10 ml., respectively. This antiserum was known to have no anti-complementary effect. Blood cultures taken from these and 2 control rabbits after injection of the organisms gave the following results: the 2 rabbits receiving antiserum had bacteremia at 4 hours but not at 8 hours or thereafter. Meningococci were not obtained from the blood of either of the normal control rabbits in any of the cultures.

##### DISCUSSION

The nature of the agent which is responsible for the bactericidal action of normal serum for the meningococcus is not clearly understood. It

appears to resemble bactericidal antibody, as Gordon and Hoyle have shown, since it requires complement for its function, can be reactivated by the addition of complement after heating, and may be removed by absorption with specific antigen (9). However, the frequency of its appearance in presumably normal sera, the extraordinary variability in the susceptibility of different strains of the same serological group to its action, and its uniform presence in guinea pig serum for Group I organisms, might cause doubt as to its specificity as an immunological response to previous infection. This doubt is increased by certain observations made in this investigation: that fresh undiluted sera from immunized animals and convalescent human patients may be completely lacking in bactericidal action, and the addition of immune serum to normal serum results in the inhibition of the bactericidal action of the latter. An explanation for all these observations would require a more complete knowledge of the prozone phenomenon than is now available, although the prozone alone is apparently responsible in some instances. Moreover, it seems evident that the presence of bactericidal action in fresh, undiluted serum is not a reliable index of the formation of specific antibody.

On the other hand, it has been demonstrated that a very high order of bactericidal antibody is present in immune sera and can be easily detected if the proper conditions are present. These conditions are (1) the strain of meningococcus must be selected on the basis of its susceptibility to bacteriolysis. If a generally susceptible strain is employed, bactericidal antibody can be demonstrated in the presence of a non-bactericidal complementing serum. If a resistant strain is used, a difference between the effectiveness of complement from different species becomes apparent, *i.e.*, rabbit complement is effective while human complement is not. At the same time, a second mechanism can be brought into action against the resistant strain, involving the use of defibrinated blood instead of serum alone; (2) the appropriate normal serum or defibrinated blood must be used as complement; and (3) the proper proportions of antiserum must be tested, in order to evade the masking effect of the prozone.

The inhibitory effect of antiserum upon the bactericidal action of normal serum *in vitro* is a

finding which is consistent with the apparent lack of bactericidal action of the sera of actively and passively immunized rabbits. There are two possible factors which may be involved in these events: (1) the prozone phenomenon, and (2) the inhibition of complement. It has been shown that either factor may produce the same end result. In the *in vitro* experiments with rabbit antisera, in which untreated monovalent sera were employed, and in the *in vivo* experiments with horse antisera, it was established that no diminution in hemolytic complement was involved in the inhibitory phenomenon. On the other hand, the results with concentrated rabbit antisera, which produced not only inhibition of bactericidal action when injected intravenously but also the persistence of bacteremia, were probably due both to the prozone phenomenon and to the anticomplementary effect of the antiserum. The reasons for this anticomplementary effect are not known. The disturbance in complement seems to have been profound, in view of the long duration of the effect. The concomitant loss of bactericidal action and hemolytic complement, which was observed following the injection of concentrated normal globulin, provides further evidence of the necessity for complement in the bactericidal mechanism.

The results of passive immunization of human subjects with concentrated rabbit antiserum are in agreement with the *in vitro* findings in bactericidal tests. It will be noted that the bactericidal property already present in serum for the susceptible strain was reduced, while killing of the resistant strain occurred in defibrinated blood, and no killing of the latter strain occurred in serum alone.

For clinical purposes, the observations reported here are of interest in a number of respects. First, it is evident that excessive amounts of antiserum may reduce, rather than enhance, the bactericidal property of the blood. Whether such a process can take place in the blood of a patient undergoing serum treatment remains to be determined and evaluated. Second, the loss of bactericidal action which was observed in highly immunized animals suggests that a similar process may be a factor in the development of prolonged or chronic infection with the meningococcus, such as chronic meningococcemia, and the bacteremia

with endocarditis which is sometimes seen in immunized horses (10). Third, a considerable anti-complementary effect may be exerted in rabbits by concentrated antiserum. It has not been shown that this effect also takes place in humans, but the question is of such practical importance that it should be studied further.

#### SUMMARY

(1) Differences in the general susceptibility of strains of Group I meningococci were associated with differences in the behavior of these organisms in the bactericidal test with immune sera. Susceptible strains were killed, while resistant strains survived, in the presence of human complement. Resistant strains were killed in the presence of rabbit complement or human defibrinated blood.

(2) Fresh immune sera were lacking in bactericidal action for both resistant and susceptible strains. When these sera were diluted in the presence of an appropriate neutral complement, strong bactericidal antibody was demonstrable.

(3) The bactericidal action of fresh normal serum was inhibited by the addition of specific antiserum. This inhibition was not associated with detectable fixation of hemolytic complement. It is considered to be a manifestation of the prozone phenomenon.

(4) The injection of small quantities of specific antiserum in rabbits produced an increase in bactericidal property. Larger doses caused inhibition of killing. In some instances, this may have been due to the anticomplementary action of concentrated antiserum; in others, complement was not affected.

(5) The injection of concentrated antiserum in 2 human subjects resulted in the enhancement of bactericidal action in defibrinated blood, for the resistant strain, and a diminution in the bactericidal action of serum and defibrinated blood, for the susceptible strain. No detectable alteration of complement was observed.

(6) The intravenous injection in rabbits of a concentrated rabbit antimeningococcal serum resulted in the disappearance of detectable hemolytic complement for as long as 24 hours. After

the intravenous injection of meningococci, bacteremia persisted for a considerably longer period in rabbits receiving antiserum than in control rabbits. Similar results were obtained using an antimeningococcal horse serum, free from any demonstrable anticomplementary effect. Two factors, operating singly or together, appear to be responsible for these results: the prozone phenomenon due to antibody excess, and anticomplementary action.

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# RENAL BIOPSY STUDIES CORRELATED WITH RENAL CLEARANCE OBSERVATIONS IN HYPERTENSIVE PATIENTS TREATED BY RADICAL SYMPATHECTOMY<sup>1</sup>

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The morphologic changes in the kidneys of patients who have died from essential hypertension and its complications are well known. The structural pattern at death is informative, but it offers little clue as to the intervening degenerative processes responsible for the terminal picture. A noteworthy effort has been made in recent years to fill the void. Various investigators (1, 2, 3) have applied the ingenious renal clearance procedures devised by Smith (4) to hypertensive patients and have obtained consistent and definite data. Anatomical studies also have been pursued in living patients, and recently one of us (B. C.) reported a small series of renal biopsies, taken during life from patients with various degrees of essential hypertension (5). The specimens were obtained during operation for sympathectomy, and all showed diffuse vascular disease. Since this preliminary report the number of biopsies has been increased to more than 100.<sup>2</sup> No attempt was made at that time to consider anatomic findings in relation to renal clearance data or to the clinical status. In the first portion of the present communication, such an attempt has been made, and the microscopic appearance of renal tissue from 20 living patients has been correlated with the function of the kidneys. The quantitative clearance procedures include measurement of rate of formation of glomerular filtrate, renal plasma flow, and maximal capacity of the tubules to excrete diodrast.<sup>3</sup> The second portion of the paper deals

with renal clearance observations as affected by sympathectomy.

## CORRELATION OF RENAL BIOPSIES AND RENAL CLEARANCE DATA

The clinical status of each of the 20 patients conformed to the type usually associated with arterial hypertension. Headache, dyspnea, vertigo, blurring of vision, nausea, vomiting, frequency of urination, nocturia, precordial pain, palpitation, paresthesia, faintness, weakness, and nervousness were frequent complaints. The systolic blood pressure usually was over 200 and the diastolic over 100 mm. Hg. The majority had evidence of hypertensive heart disease as suggested by the electrocardiogram and the measurement of heart size by x-ray. The patients were equally divided in regard to sex. Five of the females gave a history of having had a kidney disturbance or elevations of blood pressure during one or more pregnancies. Two males gave a history of having had test. Most urine specimens were collected by a soft rubber ureteral catheter. Levels of mannitol and inulin in the plasma were maintained at approximately 125 mgm. per 100 cc. The plasma level of diodrast at low levels varied between 1 and 2 mgm. per 100 cc. At high plasma levels for determination of TmD, it ranged from 40 mgm. to 25 mgm. per cc. The duration of the average collection period was 10 minutes. Blood samples for determination of concentration of clearance constituents in the serum were taken at the half-way time in many periods. When this was not deemed necessary, the concentrations were interpolated from data collected during periods immediately before and after. Venous bloods were taken at all times. A stop-watch was used for timing. The observations on glomerular filtration, renal plasma flow, and the effective renal blood flow are the mean for 4 or more 10-minute collection periods. The observations for TmD are the mean of 3 10-minute periods. All the data are corrected for a body surface area of 1.73 sq.M. The determinations were carried out with the patients in the horizontal position in a quiet room where the temperature was 70° F.

<sup>1</sup> This investigation was aided in part by a grant from the Corn Industries Research Foundation.

<sup>2</sup> A report on the microscopic findings in the first 100 cases will be made separately by two of us (B. C.; R. H. S.) J. A. M. A. (In press.)

<sup>3</sup> The clearance studies were performed by two of us (J. H. T. and R. S. M.) according to methods described by Smith and associates (4, 6). The patients were prepared for the test by allowing them to consume one liter of tap water, 12 and 2 hours, respectively, before each



cystitis. Three patients had had a cerebral hemorrhage before admission. The duration of symptoms attributed to hypertension varied from 6 months to 14 years.

Particular attention was given to the examination of the ocular fundi. The eyeground findings were graded 1 through 4; the higher the grade, the more extensive the vascular damage observed. Fifteen showed grade 2, 3, or 4.

Clinical tests of renal function included ability to concentrate solids following abstinence from fluid for 12 hours, urinary excretion of phenolsulfonephthalein dye 15 minutes after 1.0 cc. had been given intravenously, determination of concentration of non-protein nitrogen in the serum, and intravenous pyelography. These procedures gave results which were interpreted usually as consistent with unimpaired renal function. None had an elevation of the non-protein nitrogen in the serum, nor was the specific gravity of the urine fixed at a low level. All except 2 were able to concentrate to 1.020. Five were unable to excrete 25 per cent or more of phenolsulfonephthalein dye in the first 15 minutes after the intravenous injection. The examination of the urinary sediment, however, showed casts or blood cells in a preponderant number, while nearly half of the patients showed albuminuria.

An extensive removal of the sympathetic chains by one of us (R. H. S.) was performed at operation (7). In most patients, the lower 4 dorsal ganglia and the upper 1 or 2 lumbar ganglia were excised as well as a splanchnic denervation, removing the great splanchnic nerve from the semi-lunar ganglion to the mid-thoracic level. The extensive retroperitoneal exposure permitted access to the kidney and the opportunity to excise a portion for study. The specimens taken were 6 to 7 mm. wide and 5 mm. deep. Specimens were taken from both kidneys in a few patients. The gross appearance of the kidneys, when fully exposed at operation, was not remarkable. Usually, the size was normal and only rarely was the capsule firmly adherent to the renal cortex. A few minute scars were visible in approximately one-third of the cases, while the remainder of the parenchyma was smooth and appeared normal.

The 20 biopsies were graded 0, I, II, III, IV by one of us (B. C.), according to the severity of the vascular lesions. The criteria for these grades

are described in more detail in a study of the large series of 100 renal biopsies of hypertensive patients.<sup>4</sup> A valid objection might be raised that a biopsy is not an adequate sample of the whole kidney. However, great care was taken in each case to select a representative piece for biopsy, and in 25 patients out of the larger series of 100, specimens removed from both kidneys were of the same grade. It is noteworthy that not all specimens graded I and II appeared abnormal at casual inspection. Careful search, however, showed vascular changes in all 20 patients except Nos. 19 and 20, which were graded 0. The vessels were divided into three groups: (1) small arteriole, the external diameter of which measured up to 25 microns; (2) large arteriole, from 25 to 50 microns; and (3) small artery, greater than 50 microns. The last group rarely exceeded 100 microns, since the specimens were from the peripheral portions of the cortex. The vascular lesions could be classified readily under the three terms employed by Moritz and Oldt (8): intimal hyalinization, medial hypertrophy and degeneration, and endothelial hyperplasia. No necrotizing arteriolitis was observed, confirming the theory that this lesion is a terminal one. Most of the specimens showed combinations of these types, but in a few, one type was predominant. No specific type of process was limited to any one of the different sized vessels, although, by and large, the arteries showed endothelial hyperplasia with reduplication of the internal elastic lamella or fibrous intimal thickening, and the arterioles showed either medial hypertrophy or intimal hyalinization or very frequently both processes. Although the biopsy grades were based solely on the severity of the arterial and arteriolar disease, it is interesting to note that a large proportion of the glomeruli appeared normal in most of the cases. Glomerular changes were entirely absent throughout the biopsied material of kidneys classed under grade I. Some slight thickening of the capillary walls and an occasional sclerosed glomerulus were seen in the kidneys of grade II; there was otherwise little change in the glomerular tufts. In the more advanced cases, grades III and IV, often associated with visible scarring, the glomeruli adjacent to or within the scarred area were partially or com-

<sup>4</sup> See footnote 2.

pletely hyalinized. Careful examination was made of the juxta-glomerular group of cells (9) for evidence of hyperplasia, but no abnormality was observed. There were 3 patients classified in grade IV, 8 patients in grade III, 3 patients in grade II, 4 patients in grade I, and 2 patients in grade 0. Renal vascular lesions have been so well illustrated in Moritz and Oldt's paper (8), that it was felt unnecessary to include photomicrographs in this report.

*Grade IV renal vascular disease.* Advanced vascular disease in both arteries and arterioles was present in the specimens removed from 3 patients. The average glomerular filtration rate and renal plasma flow were 64 cc. and 283 cc. per minute, respectively (Table I). This represents a 50 per cent depression below the normal mean filtration rate, and a 60 per cent depression in renal plasma flow. Patient No. 2 died of a cerebral hemorrhage and uremia before discharge from the hospital.

TABLE I  
*Correlation of renal clearance observations with renal biopsies \**

Patient number	Age	Blood pressure	Retinal changes	PSP excretion in first 15 minutes	Glomerular filtration rate	Plasma flow	Effective whole blood flow †	Diodrast iodine Tm	Filtration fraction
			grade		cc. of plasma cleared per minute			mgm. per minute	per cent
GRADE IV RENAL VASCULAR DISEASE									
1	46	208/138	3	10	56	220	370	19	25
2	36	210/142	3	10	67	250	420		27
3	50	230/110	2	35	69	380	680		18
				Average	64	283	457		23.3
GRADE III RENAL VASCULAR DISEASE									
4	38	190/140	4	25	86	400	660		21
5	34	175/135	1	40	90	480	840		19
6	27	225/145	1	25	70	420	780		17
7	34	205/120	3	30	86	430	770		20
8	34	220/140	3	40	71	320	570		22
9	45	205/112	2	20	105	500	850		21
10	30	215/145	1	45	86	450	760	46	19
11	35	230/120	1	35	121	510	720	41	24
				Average	89.4	438.8	743.8	43.5	20.4
GRADE II RENAL VASCULAR DISEASE									
12	48	204/116	2	25	100	450	750	37	22
13	38	180/100	2	35	90	490	880	42	18
14	40	180/110	1	20	83	470	800	43	18
				Average	91	470	810	40.7	19.3
GRADE I RENAL VASCULAR DISEASE									
15	23	163/114	1	45	127	700		67	19
16	37	148/98	2	38	114				
17	32		1	40	100				
18	56	200/126	1	20	76	410	710	45	19
				Average	104.2	552		56	19
GRADE 0 RENAL VASCULAR DISEASE									
19	39	144/122	1	40	92	520	990		18
20	18	140/100	3	40	96	730	1210	42	13
				Average	94	625	1100		15.5

\* The 20 cases are divided into 5 groups according to the grade of renal vascular disease found in the kidney biopsies taken at operation. The renal clearance data are given for the patients in each group.

† Calculated from renal plasma flow by taking hematocrit into account.

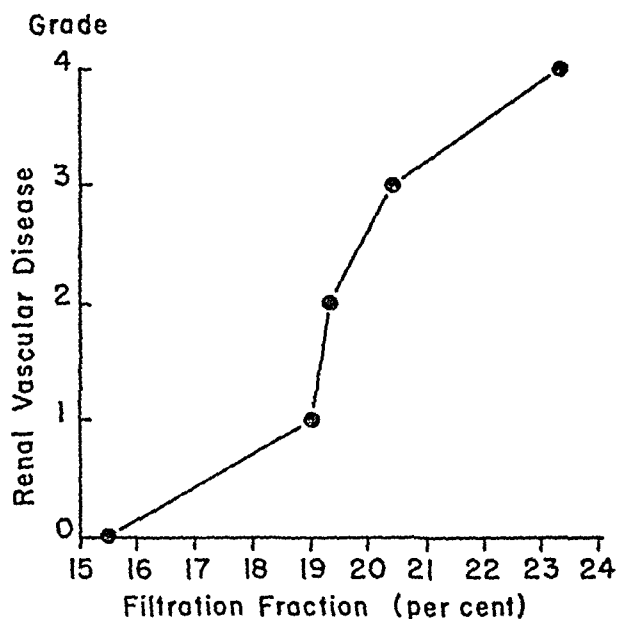


FIG. 1

The average of the filtration fractions for the cases in each biopsy group is plotted against the grade of renal vascular disease. The curve indicates that the filtration fraction increases as does the evidence of renal vascular disease, but was not above the normal of 20 per cent in the lower biopsy groups. The filtration fractions for each case are given in Table I.

This is the only patient reported in this communication who has died.

*Grade III renal vascular disease.* There were 8 patients in this group. The intimal hyalinization of the large and small arterioles in this group was similar to that in grade IV, while the arterial changes and parenchymal scarring was slightly less severe. Approximately 10 per cent of the glomeruli were sclerosed, although in some specimens no hyalinized glomeruli were seen after a careful search. The glomerular filtration rate averaged 89 cc. per minute, and the renal plasma flow, 439 cc. per minute (Table I). Both of these values represent about a 30 per cent depression below the normal.

*Grade II renal vascular disease.* The 3 cases in this group showed moderate vascular changes of all types, but especially intimal hyalinization. The average glomerular filtration rate, 91 cc. per minute, was only slightly below the average range for normals, while plasma flow, 470 cc. per minute, was depressed significantly (Table I).

*Grade I renal vascular disease.* There were 4 patients in this group. Slight but definite vascular disease was noted. Glomerular filtration rate in all except patient No. 18 was normal, although

plasma flow was depressed slightly (Table I). Patient No. 15 showed high normal values for both functions, the highest observed in any patient reported in this communication. These figures were checked at subsequent examinations and the grade I anatomical changes were confirmed. The average glomerular filtration rate and plasma flow were 104 and 552 cc. per minute, respectively.

*Grade 0 normal renal vascular findings.* The 2 patients in this group, Nos. 19 and 20 (Table I), had renal clearance data that were at, or slightly below, the lower limit of normal. Nothing was noted preoperatively to suggest an adrenal tumor as being responsible for the hypertension. Paroxysmal episodes were absent in both patients. Nevertheless, patient No. 19 had an adrenal cortical tumor and patient No. 20 had an adrenal medullary tumor. The tumor was on the right side in both patients. That these 2 instances are not unique may be assumed from the observation of 5 additional cases in the larger series of 100 biopsied cases, 4 exhibiting a cortical tumor and the other a medullary tumor.<sup>5</sup> Equally interesting is the fact that the renal vessels were normal in both patients, the only patients who showed no renal anatomical changes in this small series.<sup>6</sup> A good clinical result followed unilateral sympathectomy and excision of the tumor in patient No. 20. Patient No. 19 experienced partial relief from symptoms. He returned 4 months after the first operation for a sympathectomy on the intact side. Renal clearance observations at this time checked remarkably well with those obtained at the first admission.

*Filtration fraction.* Goldring and associates found that patients with hypertension have a greater percentile depression in renal plasma flow than in glomerular filtration rate, so that the ratio,  $\frac{(\text{glomerular filtration rate})}{(\text{renal plasma flow})}$ , designated filtration fraction by Smith (6), is increased above the normal of 20 per cent. An increase of this fraction has been interpreted as an indication of constriction of efferent renal arterioles. The av-

<sup>5</sup> These 7 cases will form the basis of a more detailed communication by Drs. Smithwick and Castleman.

<sup>6</sup> In the larger series of renal biopsies, there was another patient with cortical adrenal tumor and 4 patients without adrenal tumors who showed no renal vascular disease.

# RENAL BIOPSY AND CLEARANCE STUDIES IN HYPERTENSION

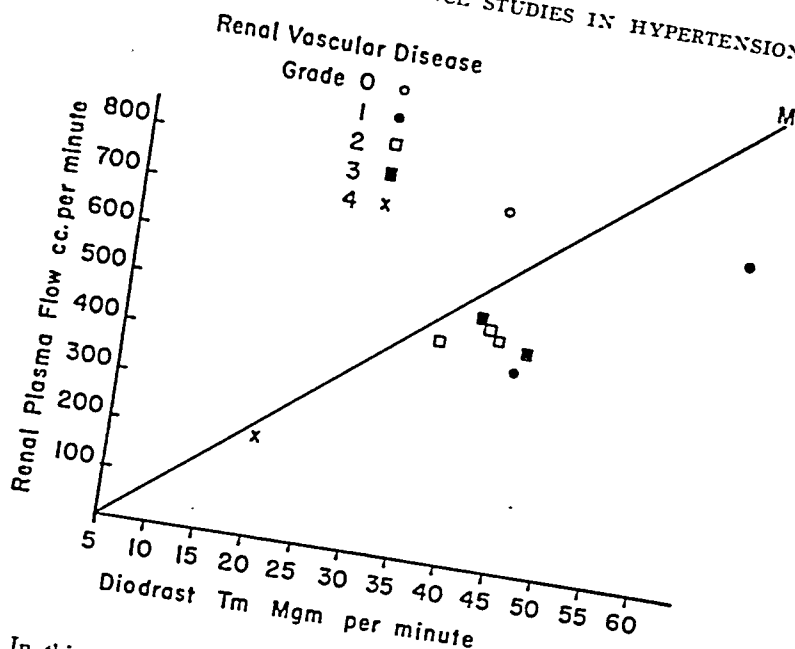


FIG. 2  
In this figure, diodrast Tm is plotted against renal plasma flow. These data are available in 9 of the 20 cases. The grade of the renal biopsy is indicated in each instance.

erage filtration fraction in our series of patients showed a slight increase, 21.6 per cent, and there was a definite trend for the percentage to decrease with the less severe renal vascular disease (Figure 1). In biopsy groups 0, I, and II, the filtration fraction was normal in 7 of 8 cases (Table I), while in groups III and IV, the filtration fraction was above normal in 6 of 11 cases.

*Diodrast Tm* (maximal capacity for excreting diodrast iodine) was determined in approximately half of the patients (Table I). If 45 mgm. per minute is assumed to be the lower level of the normal, only 3 patients had a normal value and the percentile depression followed closely the depression in glomerular filtration rate. In Figure 2, renal plasma flow is plotted against TmD, according to Goldring. All of the data except one are below line M, which is drawn so as to represent a remass of proportionate amounts. That is, if some process were operating to reduce plasma flow and tubular excretory mass proportionately, the regression would follow line M. In a heterogeneous group of renal disorders, there should be a scatter of data with points above line M as well as below. Inspection of Figure 2 shows that some factor is operating in hypertensive subjects to produce a

relative ischemia in the residual functioning tubular tissue.

## RENAL CLEARANCE OBSERVATIONS FOLLOWING SYMPATHECTOMY

Renal clearance observations following sympathectomy were obtained in 3 types of patients. (1) In 9 patients, the observations were obtained within 2 weeks after the second stage sympathectomy for comparison with the studies before operation. (2) In 9 patients, the studies were obtained from 4 to 13 months after the second operation for comparison with studies both before and within 2 weeks after operation. (3) In 6 patients, no preoperative or immediately postoperative studies were obtained, but they were made from 18 months to 4 years following operation.

Inspection of Table II shows that the glomerular filtration rate decreased about 20 per cent during the immediate postoperative period, but within a year had returned to about its preoperative level. Although there were no preoperative studies on the 6 patients that were followed from 1½ to 4 years, their postoperative filtration rates did not differ materially from the preoperative values of the other cases. It seems reasonable to con-

TABLE II  
Effect of sympathectomy on renal clearance \*

Patient number	Renal biopsy (Grade)	Glomerular filtration rate				Plasma flow				Effective whole blood flow				Diodrast iodine Tm				Filtration fraction			
		Pre-operative	Postoperative			Pre-operative	Postoperative			Pre-operative	Postoperative			Pre-operative	Postoperative			Pre-operative	Postoperative		
			With-in 2 weeks	4 to 13 months	1½ to 4 years		With-in 2 weeks	4 to 13 months	1½ to 4 years		With-in 2 weeks	4 to 13 months	1½ to 4 years		With-in 2 weeks	4 to 13 months	1½ to 4 years		With-in 2 weeks	4 to 13 months	1½ to 4 years
2	IV	67	26			250	110			420	170							27	24		
8	III	71	73			320	320			570	520							22	23		
10	III	86	45			450	270			760	430							19	17		
13	II	90	83			490	540			880	860			46	20			18	15		
4	III	86	58	104		400	340	370		660	580	630		42	45			21	17	28	
5	III	90	91	94		480	630	290		840	1020	480						19	14	32	
6	III	70	92	92		420	500	480		780	740	800			39			17	18	19	
7	III	86	71	55		430	440	300		770	660	540						20	16	18	
9	III	105	84	90		500	420	270		850	440	440			38			21	20	33	
1	IV	56		56		220		240		370		385		19	21			25		25	
12	II	100		118		450		400		750		670		37	42			22		27	
15	I	127				700		760						67				19			
21		91				350		170		620				49				26			
22	II				59				250				410								24
23	III		93	57	64		300	290	230		530	470	410		43	28		31	49	20	28
24					98																
25					74				370				600								20
26					96				400				680								24
27					76				320				540			39					24

\* The renal clearance data are arranged under 5 headings: glomerular filtration rate, plasma flow, effective whole blood flow, diodrast Tm, and filtration fraction. The relation to the time of operation is indicated in each instance. The number of the patient and the biopsy grades are given. (Refer to Table I.)

therefore, that glomerular filtration rate is unaltered by sympathectomy. In only 1 patient, No. 4, was a below normal rate before operation restored to normal by operation.

The renal plasma flow studies did not show any significant change in the immediate postoperative period, but did decrease about 17 per cent within a year and remained approximately the same for the next few years.

The filtration fraction improved somewhat during the 2-week period after operation, but gradually rose during the first year and showed no tendency to return to normal later. This finding suggests that efferent constriction had not been lessened by sympathectomy.

The diodrast Tm observations are too few and scattered to warrant any conclusions.

#### DISCUSSION

From these data, admittedly few for the malady under discussion, it is evident that in patients with essential hypertension a reasonably constant correlation exists between microscopic evidence of renal vascular disease and renal function as measured by quantitative procedures. A study of Figures 3 and 4 suggests that the reduction in glomerular filtration, and especially in renal blood flow, increases as the vascular disease progresses.

Furthermore, it is of interest to note that, in the absence of renal vascular disease (grade 0), or in the presence of minimal arteriolar changes (grade I), the reduction in renal blood flow is slight, if any. It is only in the grade IV biopsy group that renal blood flow is reduced to a serious level. Ex-

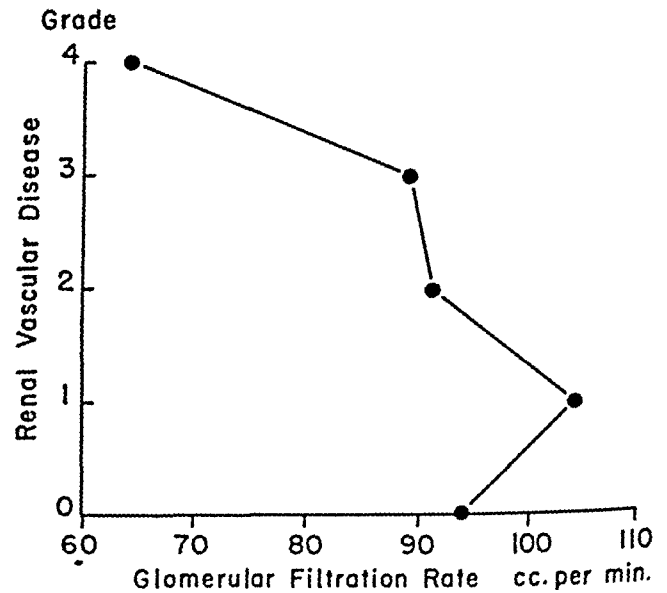


FIG. 3

In this figure, the glomerular filtration rate is plotted against the grade of renal vascular disease. It indicates that the filtration rate decreases as the vascular disease increases. It varies from the normal range to serious reduction, the latter being found only in the grade IV biopsy group.

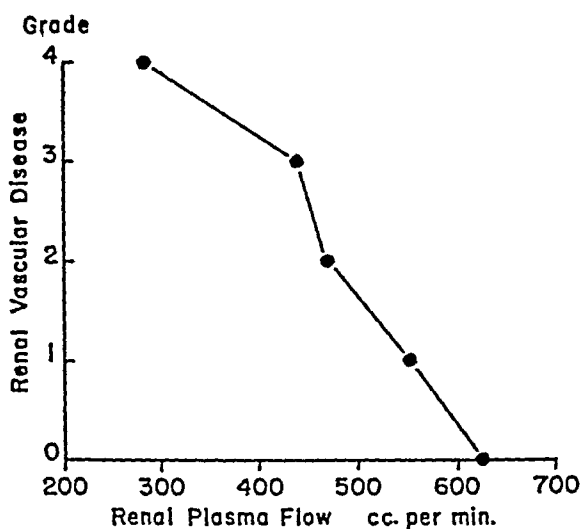


FIG. 4

In this figure, the renal plasma flow is plotted against renal vascular disease as judged by the kidney biopsies. The renal blood flow decreases as the renal vascular disease increases. The range is from the normal level to a marked reduction, the latter being found in the grade IV biopsy group only.

ceptions, however, were noted. Thus, patient No. 18 had grade I vascular disease but a marked diminution in glomerular filtration, while patients Nos. 9 and 11 had grade III vascular disease and normal filtration rates. In the main, most discrepancies, when present, suggested a greater degree of vascular disease than was actually present. This is to be contrasted with ordinary function tests which, in general, fail to indicate vascular disease until it is present in its most advanced form.

The patients in our biopsy group may be compared with a series of 60 patients with essential hypertension, reported by Goldring and associates (1), in whom only renal clearance studies were performed. These patients were not operated upon and no renal biopsies were available. Except for the 8 severe cases in Goldring's group, all but 1 of whom died before his report was published, the patients in our series are believed to have suffered from a more advanced grade of hypertension. This statement is based principally upon the greater observed depression in renal clearance. In Goldring's series, approximately 50 per cent of the patients had a glomerular filtration rate above

92 cc. per minute, while less than one-third of our patients showed as satisfactory a rate. This is emphasized because of the good clinical results that followed sympathectomy in our series. Most of the patients experienced marked clinical improvement and a recent follow-up has shown no death other than that of patient No. 2, who died in the hospital.

An increase in filtration fraction in certain hypertensive patients has been emphasized in the literature as it indicates constriction of the efferent glomerular arterioles. The cause of this constriction is unknown, but it has been interpreted by some to result from the action of a circulating humoral pressor substance. It is of interest to note that in the early stages of renal arteriolar disease, biopsy groups 0 and I, the filtration fraction was normal in 4 of 4 cases. In grade II, it was normal in 2 of 3 patients. In grades III and IV, it was above the normal level in 6 of 11 cases. This suggests that efferent constriction is not present in the earlier stages of renal arteriolar disease.

In order to reduce to a minimum other causes of modified renal blood flow, particularly that due to vasoconstriction, the blood flow determinations both before and after operation were made with the patient in the horizontal position in a comfortable quiet environment. Studies made under other conditions, designed to bring out the effect of vasoconstriction, have been made in other patients and will be reported separately.

There was no significant difference in filtration rate or renal plasma flow following bilateral sympathectomy, after circulatory adjustments had been made.

It is most important, however, to emphasize the fact that glomerular filtration rate tends to be maintained soon after operation, as well as years later, and it may be assumed that renal damage may not progress rapidly thereafter. If this be the only effect of sympathectomy in essential hypertension, it is well worth the surgical risk and effort involved. Patients with a moderate degree of impairment of glomerular filtration (70 to 100 cc. per minute) are not seriously handicapped and it is conceivable that if their kidneys are able to escape further damage, life may not be materially shortened by the vascular changes that have developed up to the time of operation.

## SUMMARY

(1) Renal clearance studies performed on 20 patients with essential hypertension showed a significant correlation with the microscopic appearance of their respective renal tissues which were removed for biopsy at the time of sympathectomy, *i.e.*, the more severe the renal vascular disease, the more reduced were the glomerular filtration rate and the renal blood flow. In the cases with grade 0 and I renal vascular disease, the renal clearance observations were either normal or only very slightly reduced. Only in grade IV renal vascular disease was renal blood flow seriously reduced.

(2) The filtration fraction was normal in 7 out of 8 cases in biopsy groups 0, I, and II. It was increased in 6 of 11 cases in biopsy groups III and IV. These findings indicate that constriction of the efferent glomerular arterioles was not present in the early stages of renal vascular disease.

(3) Bilateral radical lumbo-dorsal splanchnicectomy had relatively little effect on renal clearance, when measured in the horizontal position. Although glomerular filtration was reduced in the immediate postoperative period about 20 per cent, within a year it returned to and continued to maintain its preoperative level. Renal plasma flow was essentially unchanged.

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# A QUANTITATIVE STUDY OF THE URINARY EXCRETION OF HYPOPHYSEAL GONADOTROPIN, ESTROGEN, AND NEUTRAL 17-KETOSTEROIDS OF NORMAL MEN

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The urinary output of hypophyseal gonadotropin by normal women has proven exceedingly variable from month to month (1, 2). Thus, the usefulness of gonadotropin assays for clinical purposes has been disappointing and is now limited to the demonstration of large excesses of hormone.

The status of the gonadotropin assay as a potential diagnostic measure in men is still in doubt. More normal data are needed to detail the normal range of excretion and to settle the disagreement between Harris and Brand (3) and Heller, Heller, and Severinghaus (4), concerning the presence of a cycle in men. Accordingly, the present study was undertaken. Concurrent assays of estrogens and neutral 17-ketosteroids were done to determine any correlation with gonadotropin output. The output of these sex hormones follows the pattern described by Gallagher, *et al.* (5), except that in their studies androgens were assayed in capons.

## MATERIALS AND METHODS

All urine specimens were complete 48-hour collections, kept in cork or glass stoppered bottles, containing 10 to 15 cc. chloroform. The urine was placed in contact with the preservative immediately on voiding. In most instances, the bottles were kept in the cold during collection. The chloroform is important since it limits the toxicity of the gonadotropic extracts prepared by tannic acid precipitation, without interfering with the colorimetric reaction for 17-ketosteroids. A rubber stopper is probably not desirable since chloroform can dissolve material from the stopper which may be chromogenic in the Zimmermann reaction. The methods of extraction and assay in this study were entirely similar to those previously reported (1), with the exception of several modifications made in the 17-ketosteroid reaction, to conform with the method of Callow, Callow, and Emmens (6), as detailed elsewhere (7). The final color was read in an Evelyn colorimeter, using filters at 520 and 440 millimicrons to check the purity of each extract (8). Hypophyseal gonadotropin was prepared by tannic acid precipitation and was assayed by the mouse uterine weight method of Levin

and Tyndale (9). Estrogen assays were conducted by the method of Kahnt and Doisy (10) on tested castrate rats. To insure uniformity of response, animals from a special colony of mice of the Swiss strain and rats of the Long-Evans strain were used. The mice were repeatedly tested for sensitivity to urinary gonadotropins with a constant weight of a castrate urine preparation, furnished by Dr. Louis Levin of the Anatomy Department; the rats were standardized for estrogen response with crystalline estrone.<sup>1</sup> Except for a definitely increased sensitivity in the summer months, both groups of animals showed a uniform response throughout the year. The mouse unit for gonadotropin was not considered positive unless 2 of 3 mice showed uterine weights greater than 0.0135 gram at autopsy. This weight was never seen in control mice with initial body weights of less than 12 grams at 22 days of age, and then autopsied at the same age as the test mice. A rat unit varied between 0.4 and 0.7 gamma of estrone, averaging about 0.6 gamma. This average was slightly less than that of the same colony during 1939 and 1940. Extracts were generally kept in the ice-box in a dry state, tightly stoppered, and for no more than 3 weeks before dissolving for assay. No significant change in potency has been noted to occur with storage of other specimens up to 6 months.

## RESULTS

The urine which was assayed was collected from 5 healthy, normal men, and was their entire output during the period of the experiment. The subjects carried on their daily routine, except that subjects N. J. and N. V. were undergoing a correlative psychologic study by Dr. George Daniels, of the Department of Psychiatry, during the period of the experiment. All remained healthy. The ages were 21, 22, 23, 24, and 33 years. Only subject N. D. was married.

The hormonal output of each of the 5 subjects is shown in Figures 1 to 5. The curves of excretion were approximately the same for all the subjects, except for the striking spurt in gonado-

<sup>1</sup> Dr. Edward Henderson of the Schering Corporation furnished the crystalline estrone used in this study.



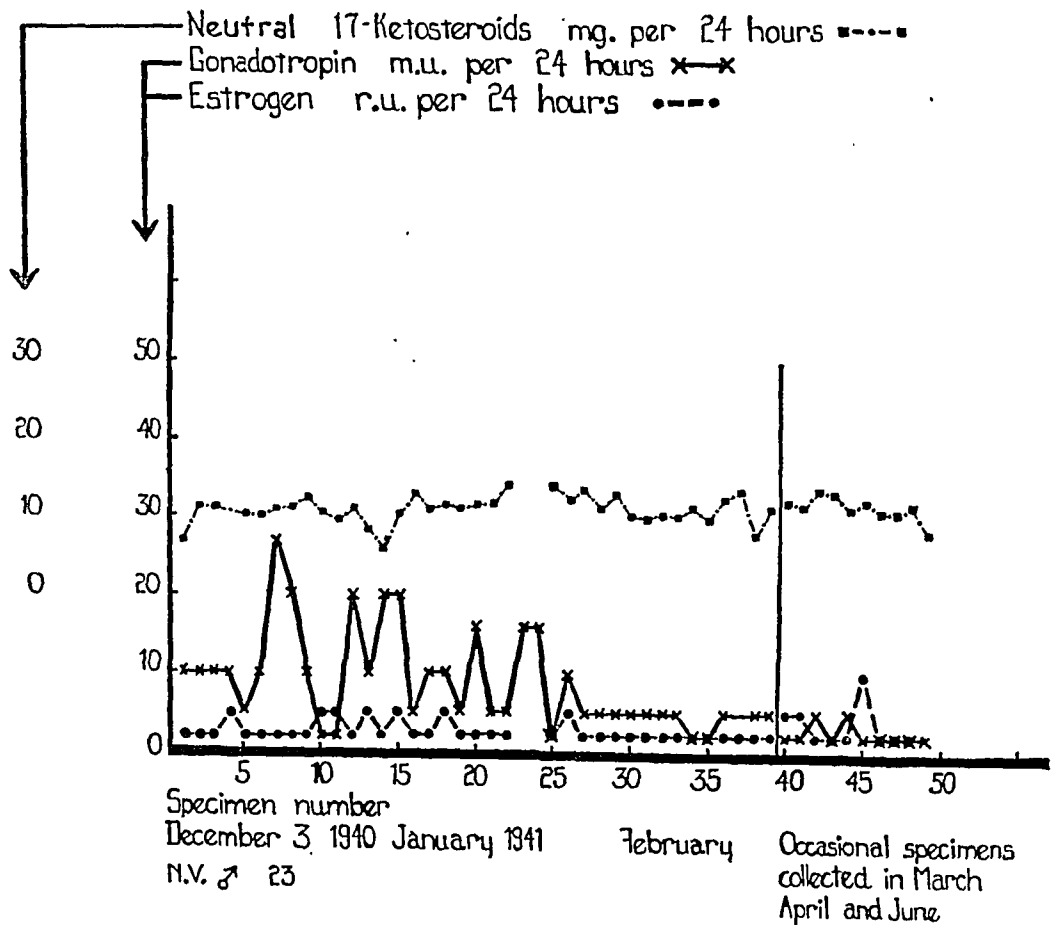


FIG. 1. FORTY-EIGHT HOUR EXCRETION OF GONADOTROPIN, ESTROGEN, AND 17-KETOSTEROIDS IN A NORMAL MALE, SHOWING A SHARP DECLINE IN GONADOTROPIN AND ESTROGEN EXCRETION IN THE LATTER HALF OF THE STUDY. SUBJECT N. V.

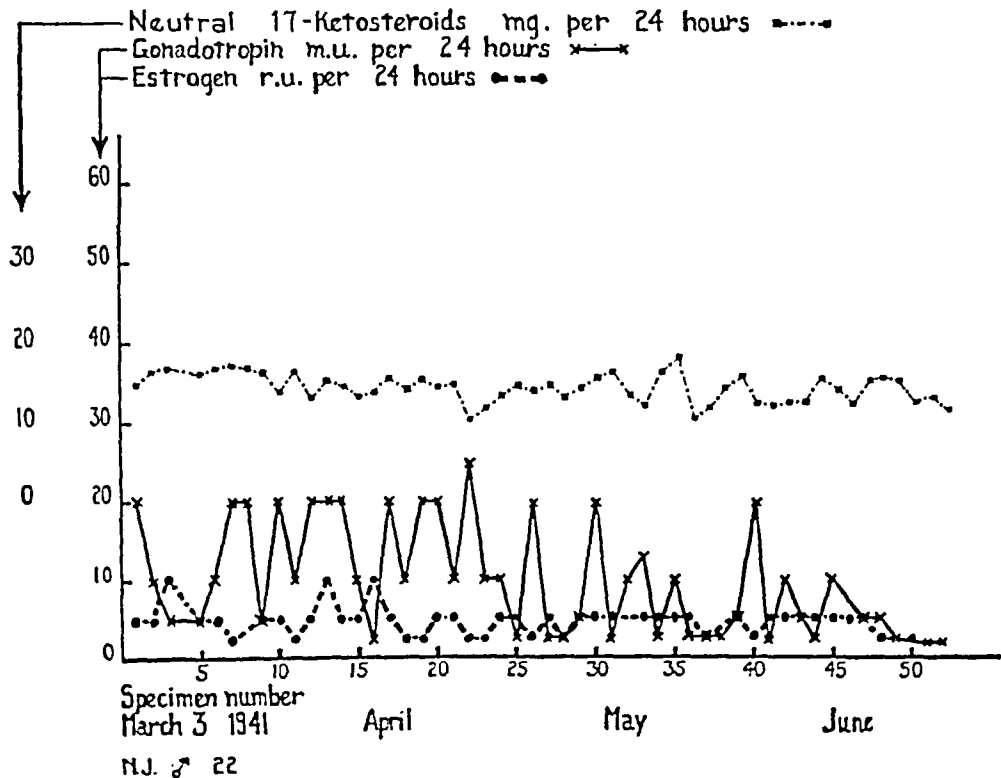


FIG. 2. FORTY-EIGHT HOUR EXCRETION OF GONADOTROPIN, ESTROGEN, AND 17-KETOSTEROIDS SHOWING A DECLINE IN GONADOTROPIN EXCRETION WITHOUT CORRESPONDING CHANGE IN ESTROGEN. SUBJECT N. J.

# GONADOTROPIN, ESTROGEN, AND 17 K-S IN NORMAL MEN

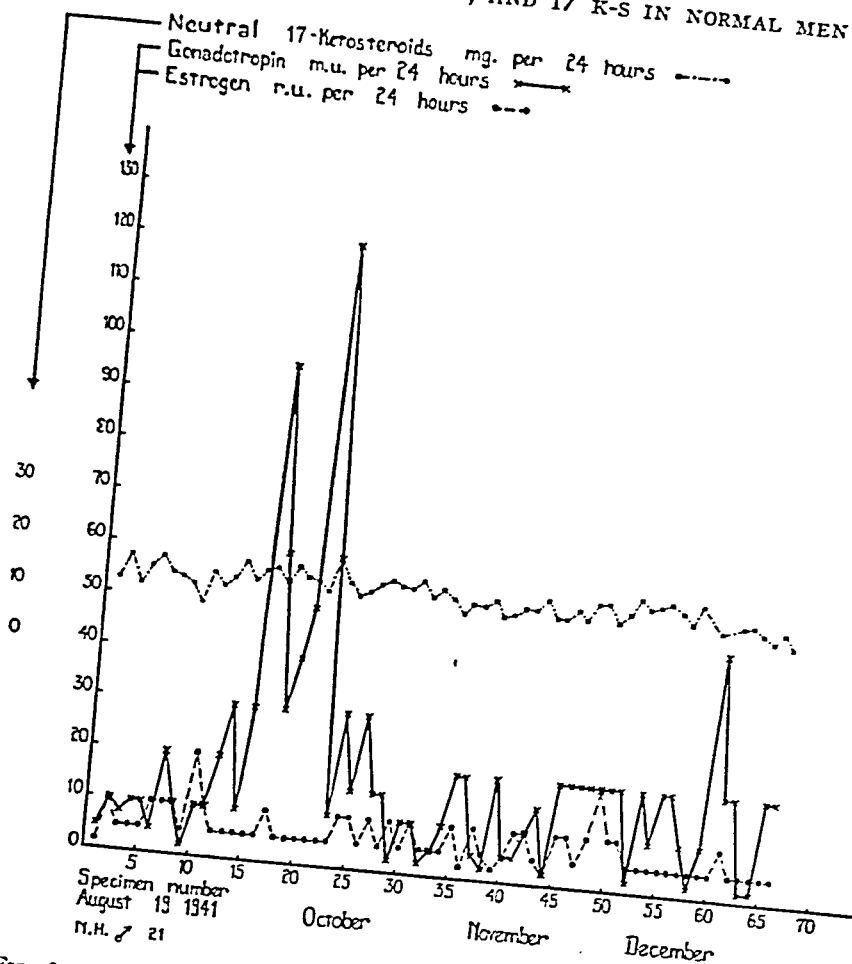


FIG. 3. FORTY-EIGHT HOUR EXCRETION OF GONADOTROPIN, ESTROGEN, AND 17-KETOSTEROIDS SHOWING A SHARP AND PROLONGED SPURT IN GONADOTROPIN EXCRETION. SUBJECT N. H.

tropin excretion shown by N. H. and the later decline in gonadotropin output of both N. J. and N. V. The average estrogen output was consistently greater in some than in others. The daily output showed extreme variability in all the subjects. The 17-ketosteroid excretion fluctuated from day to day within relatively narrow limits, but the average output varied between individuals. *Gonadotropin.* Gonadotropin was usually demonstrable in all subjects (Figures, and Table I). The daily excretion varied from less than a demonstrable amount (*i.e.* less than 5 m.u.) to as much as 120 m.u. per 24 hours. The values rarely exceeded 40 m.u. per 24 hours, however. In the figures, values between 0 and 10 m.u. mean that the assay for the higher figure was negative and could not be made for lower values because of

toxicity of some of the extracts. Thus, values below 10 mouse units are not quantitatively accurate. Over long periods, the average gonadotropin output showed great fluctuation. In subject N. V., the excretion during the first month and a half was approximately double that of the second period. Similar high or low levels, lasting several weeks, occurred in the other subjects. This was especially striking in subject N. H. There is no suggestion of periodicity or rhythmicity to these changes. The two peaks of excretion, occurring about a month apart, in subject N. D. might have been thought to indicate some periodicity as suggested by Harris and Brand (3). Because these peaks did not subsequently reappear, their significance remains in doubt. The spurt in gonadotropin excretion by subject N. H. in the first few

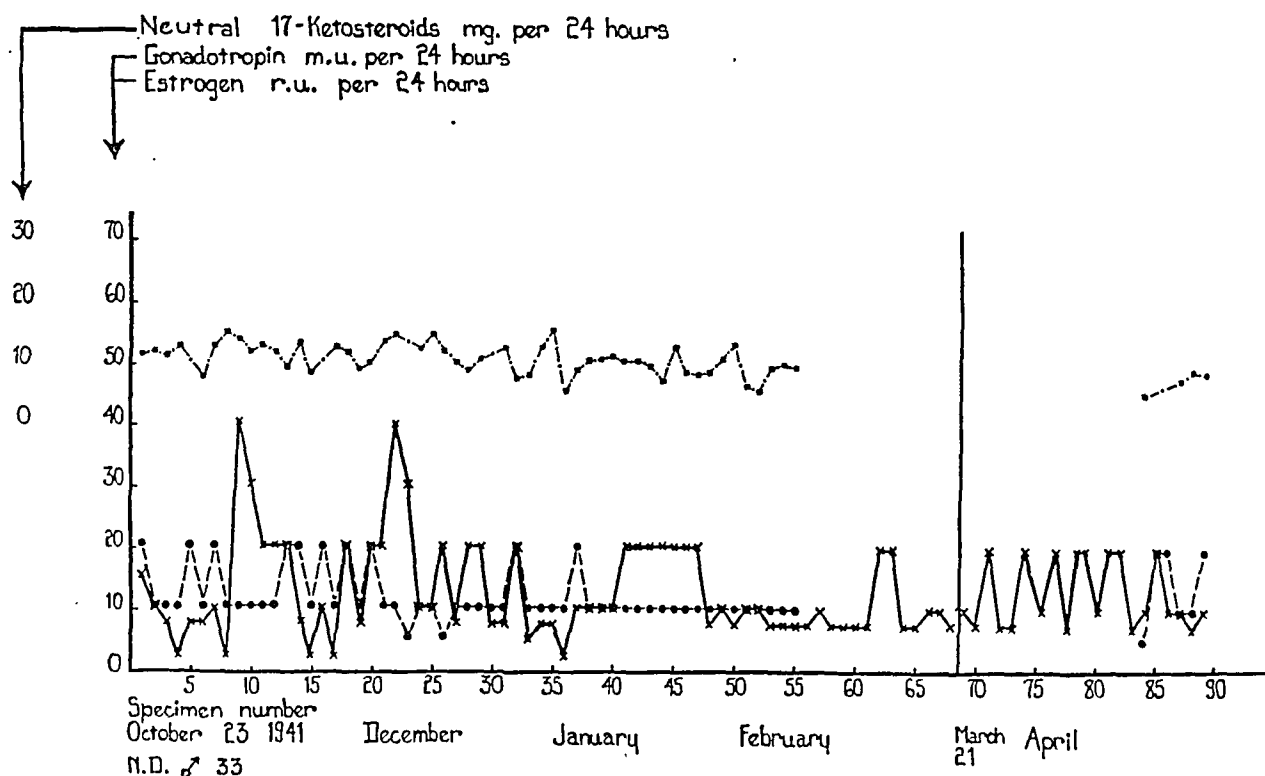


FIG. 4. FORTY-EIGHT HOUR EXCRETION OF GONADOTROPIN, ESTROGEN, AND 17-KETOSTEROIDS SHOWING FLUCTUATIONS IN GONADOTROPIN AND ESTROGEN EXCRETION OVER LONG PERIODS, AND ILLUSTRATING 2 RECURRENT PEAKS OF GONADOTROPIN. SUBJECT N. D.

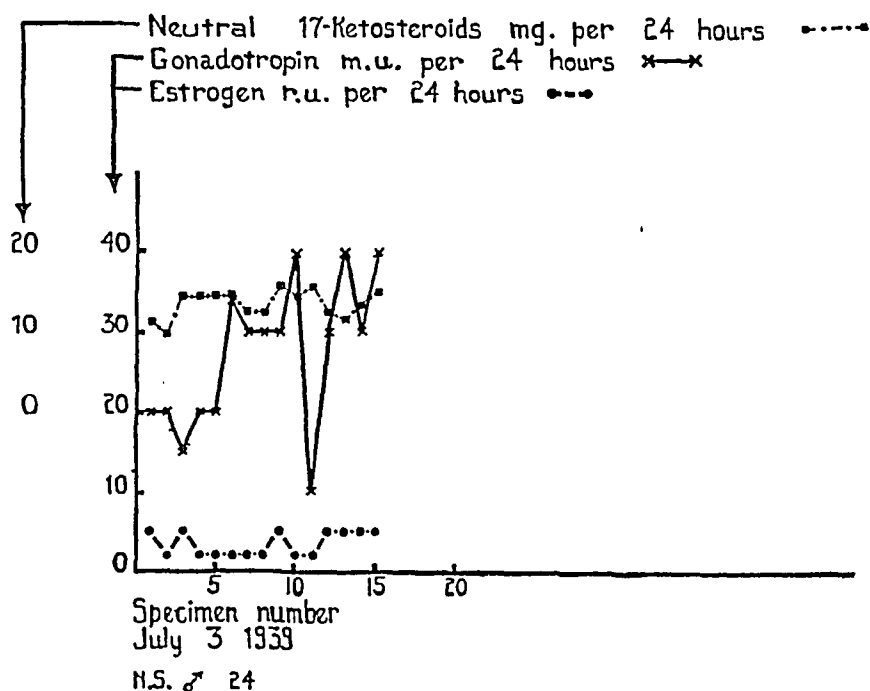


FIG. 5. FORTY-EIGHT HOUR EXCRETION OF GONADOTROPIN, ESTROGEN, AND 17-KETOSTEROIDS SHOWING A PROLONGED SPURT IN GONADOTROPIN EXCRETION. SUBJECT N. S.

weeks of the study is noteworthy and is discussed below. This sharp increase was not reduplicated in this or in the other cases.

*Estrogen.* Estrogen values represent the total of free and combined estrogen (11). No attempt

was made to separate the various estrogens. Extracts which did not give a response equal to 5 units or more are considered to be zero. The long term average excretion of estrogen differs greatly between subjects. Not only is the daily variation

TABLE I

Range of normal values for the excretion of gonadotropin, estrogen, and neutral 17-ketosteroids

Subject	Gonadotropin	Estrogen	17-ketosteroids	Estrogen-17-ketosteroid
	<i>mouse units per 24 hours</i>	<i>rat units per 24 hours</i>	<i>mgm. per 24 hours</i>	<i>ratio</i>
N. S.	10 to 40	<5 to 5	11.3 to 15.9	0.17
Average	25.3	2.3	13.6	
N. J.	<5 to 20	<5 to 10	10.1 to 18.1	0.30
Average	10.9	4.3	14.4	
N. V.	<5 to 20	<5 to 10	6.0 to 14.4	0.09
Average	8.2	0.95	11.1	
N. D.	<5 to 40	<5 to 30	5.1 to 15.2	1.14
Average	12.0	11.7	10.3	
N. H.	<5 to 120	<5 to 20	7.3 to 19.3	0.45
Average	14.8	6.4	14.3	
Range for series	<5 to 120	<5 to 30	5.1 to 19.3	0.09 to 1.14

in estrogen excretion tremendous, but the average excretion over long periods also differs. Subject N. D., for example, excreted about twice as much as subject N. H. over an equal length of time. The average output of the same individual over periods of several weeks may change, as is shown in the values obtained during the first and second halves of the assays of subject N. V. (Figure 1). The individual values for the series ranged from less than 5 r.u. to 30 r.u. per 24 hours (roughly less than 30 to 180 i.u. per 24 hours) (Table I). The average output of the various subjects varied from less than 0.95 to 11.7 r.u. per 24 hours (from 6 to 70 i.u. per 24 hours) (Table I).

*Neutral 17-ketosteroids.* The figures for 17-ketosteroid excretion for the first 2 months in subjects N. S. and N. V. were reported elsewhere to demonstrate that the day to day variation of  $\pm 40$  per cent about the mean, which normally occurs in both men and women, is due to an actual fluctuation in excretion of 17-ketosteroids (7), and not to non-specific chromogens also present in urine. In the present study, the average of the daily outputs was constant for an individual but varied between subjects. The range of excretion for the series was 5.1 mgm. to 19.3 mgm. per 24 hours. The averages for the various subjects varied between 10.3 mgm. and 14.4 mgm. per 24 hours.

#### DISCUSSION

Data have been obtained showing the quantitative excretion of gonadotropin, estrogen, and 17-

ketosteroids by 4 medically normal men, during 3 or more months, and by one man for one month. Two facts stand out from this study. (1) There is a marked and unrelated, day to day variation in output of hypophyseal gonadotropin, estrogen, and 17-ketosteroids. (2) There is a striking fluctuation in the excretion of gonadotropin over longer periods. There is no evidence of any cycle in the excretion of any of these hormones.

The reason for the fluctuation in the levels of gonadotropin is difficult to determine. The question immediately arises as to whether this fluctuation is due to a real increase in excretion of hormone as the result of a physiological change, or is due to an apparent increase in excretion caused by spontaneous variations in the sensitivity of the assay animals. The honesty of all donors seems unquestionable and there seems no likelihood of the specimens having come from some other source, either accidentally or deliberately. Periodic testing of the mice throughout the year with a standard weight of gonadotropic material, obtained from a castrate urine preparation, reveals a significant increase in the sensitivity of the mice toward the end of the summer. The sensitivity is, however, quite constant through the rest of the year (Table II). The mice were not especially sensitive when tested at the time of the very high values displayed by subject N. H. Therefore, these values appear to be real. However, the fact that the high titer did not reappear during the next 8 months was disappointing if the initial spurt is to be accounted for on a physiological basis. Whether the spectacularly high values are real or not, the fluctuations in average gonadotropin values over various periods must reflect a true physiological change in view of the occurrence of this phenomenon in all subjects. The evaluation of abnormal excretion of gonadotropin by present technics must take into account these normal variations, whether due to physiological change or the result of limitations of the biological method.

It becomes clear that the clinical value of hormonal assays in men, as in women, is subject to sharp limitations because of this variability in the normal hormonal output. A marked increase in the excretion of these hormones can be established by a few tests, but a subnormal output can be determined only after a long series of assays. Random samples over several weeks from a

TABLE II  
*Mouse uterine weight response to a constant test dose of castrate urine principle*

Date of assay	Preparation number	Number of mice	Range of uterine weight response	Vaginal response		Type response	
				Open	Closed	Number positive	Number negative
October 17, 1941	L 610 K Average	12	<i>grams</i> 0.0087 to 0.0192 0.0136	2	10	7	5
November 8, 1941	L 610 L Average	9	0.0096 to 0.0221 0.0160	0	9	7	2
February 13, 1942	L 610 M Average	9	0.0043 to 0.0263 0.0172	0	9	7	2
April 21, 1942	L 610 N Average	9	0.0117 to 0.0330 0.0198	0	9	7	2
June 5, 1942	L 610 O Average	9	0.0095 to 0.0296 0.0174	0	9	7	2
September 17, 1942	L 610 P Average	9	0.0165 to 0.0354 0.0226	6	3	9	0
October 2, 1942	L 610 Q Average	18	0.0096 to 0.0470 0.0218	8	10	15	3

normal individual (subject N. J.) revealed less than a determinable amount of gonadotropin. In establishing the presence of a disturbance which might be expected to cause a decrease in gonadotropin output, such as anterior hypopituitarism, it becomes evident that more than an occasional negative value is needed. To undertake a series of assays sharply limits the feasibility of this procedure as an aid in diagnosis. The same applies to estrogen assays. However, relatively few tests are necessary to determine an abnormal change in the 17-ketosteroid excretion.

Individual or average estrogen and 17-ketosteroid values show no correlation, either with gonadotropin output or with each other. The estrogen:androgen ratio (5), reveals a tremendous variation from day to day in the same person. Thus, it does not appear to have any value in clinical practice.

The average values for 17-ketosteroid excretion fall within the range of output of normal women (1). This does not fit with the generalization of Fraser *et al.* (12) who postulate that men excrete more than women, on the ground that an equal fraction of the 17-ketosteroid excretion is derived from the adrenal cortex in both sexes, whereas the men excrete an additional amount from the testes.

A word of discussion concerning assay methods is in order since these undergo such frequent revision. For example, the colorimetric determination of the neutral 17-ketosteroids, a more or less

standardized chemical procedure (6), has been recently extended to include fractionation of these steroids (13, 14), and this fractionation will probably be adopted for routine use. The determination of estrogen or gonadotropin, as yet a biological procedure, is in an even greater state of flux. For the estrogens, the principles of extraction are generally agreed upon, but a number of assay methods are in use (15). For gonadotropin determination, the lack of agreement includes both extraction and assay (16).

The tannic acid procedure has been used in this study in preference to the alcohol precipitation method. Assays have been possible in unpublished work for as low as 2.5 m.u. per 24 hours, and almost invariably for 10 m.u. per 24 hours. Such results are as good, if not better, quantitatively, than those of other methods. No loss of potency is indicated (17), and less toxicity has been observed. Smaller amounts of alcohol are necessary than with the alcohol precipitation method, and the method is quicker, since dialysis of the precipitate is not necessary. The tannic acid method also permits the assay of estrogen and androgen on the same specimen, which is not possible with alcohol precipitation. A possible objection is raised by Evans (18) who has stated that follicle stimulating and luteinizing fractions may not be precipitated equally by tannic acid as compared to alcohol.

A review of the control data from the mouse assays with the same preparation of gonadotropin

reemphasizes the necessity of having an adequate number of test animals to assay the extracts. The value of a one-animal assay is obviously very slight, and even a 3-mouse test may give a positive, negative, or markedly positive result with the same dosage. It is, therefore, advisable to use at least 9 mice, in groups of 3, testing the suspected unit level and a level on either side, so that a more positive, and a completely negative, group confirm the result. The other alternative is to use large numbers of animals at a given dose level. Less than 5 m.u. can rarely be tested because of toxicity, so that only 2 groups of 3 mice, for 5 and 10 units, have been employed at this unit level. In any event, a 24- or 48-hour collection is necessary to furnish enough extract to assay for these lower values. The same applies to the estrogen assays in rats. It is also advisable to use the various dose levels soon after their preparation, since aqueous gonadotropin solutions slowly lose potency. This is an important factor if several reassays are necessary, especially in the warm months.

## SUMMARY

The urinary output of hypophyseal gonadotropin, estrogen, and 17-ketosteroids of 4 normal men has been assayed over a period of 3 months or more, and of one man for a period of one month. Marked fluctuations are noted when the outputs of gonadotropin over relatively long periods are compared, and a similar variation is noted over shorter periods in estrogen and 17-ketosteroid excretion. No correlation is found between the output of gonadotropin, estrogen, and 17-ketosteroids. An increase in sensitivity of the mouse uterine test has been found to occur in the late summer. The implications of the above are discussed.

The author is indebted to Dr. P. E. Smith for his advice and to Miss E. Zabriskie and Mrs. B. Myer for technical assistance.

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# CARBONIC ANHYDRASE IN NEWBORN INFANTS<sup>1</sup>

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Carbonic anhydrase was described first in 1932 (1) as an enzyme which accelerates the reaction  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$ . Meldrum and Roughton (2) found that goat fetuses were low in carbonic anhydrase and stated, "In the very young fetuses, there is extraordinarily little enzyme and the amount does not begin to rise appreciably until very near the end of term." It was shown, further, that the enzyme is present with hemoglobin in the red blood cells (3) but is separable from these and gives the reactions of a zinc protein (4). The enzyme is inhibited by serum (5).

It has been our impression for some time that premature and full-term newborn infants who are not thriving—*e.g.*, showing poor color, feeble respirations, little or no gain in weight, low vitality—are benefited by transfusions of whole blood from a compatible donor. With the above findings in mind, determinations of carbonic anhydrase were done on newborn infants in order to determine whether infants show a significant deficiency of carbonic anhydrase which can be restored by blood transfusions.

## METHOD

The glass boat method, described by Meldrum and Roughton (2), was used. Two cc. of 0.2 M phosphate buffer (pH 6.8) were shaken vigorously with 2.0 cc. of 0.2 M sodium bicarbonate in an air tight chamber connected to a water manometer. The velocity of carbon dioxide evolution was noted. With an identical set-up, a small amount of blood was added and the increment in the velocity of the evolution of carbon dioxide was utilized as a measure of the carbonic anhydrase present in the blood. Enzyme unitage was calculated as described by these authors, with the exception that the present experiments were run at 0° C. and no correction for temperature has been made. Throughout the present work, the same machine has been used.

While for some purposes, the changes in the concentration of the blood cells would have to be taken into consideration, the changes in the enzyme concentrations in

the present observations are of such magnitude as to permit one to disregard this factor. Furthermore, in the early work, we determined the number of red cells, the concentration of hemoglobin, and the volume of red cells by hematocrit, at the same time as carbonic anhydrase determinations and found no marked correlation.

Blood was obtained by an incision of the toe or heel, adequate to insure a free flow without recourse to squeezing the tissues. It was collected in a 0.1 cc. capillary pipette and mixed immediately with 20 cc. of distilled water with a resulting 1:200 dilution. Five-tenths cc. of this dilution was added to the bicarbonate solution when running a determination.

## RESULTS

Table I gives average blood levels of carbonic anhydrase for 4 categories of normal individuals. The adults were healthy medical students and nurses. The premature infants, weighing less

TABLE I  
Normal blood levels of carbonic anhydrase

Classification	Number of cases	E per c. mm.*	Standard deviation
Adult males	11	3.51	0.360
Adult females	17	3.28	0.339
Prematures	39	0.79	0.470
Normal newborns †	56	1.41	0.371

\* In all tables, "E per c. mm." denotes units of carbonic anhydrase per cubic millimeter of blood.

† Includes first 7 days of life. Standard deviation of averages for each of these days: 0.194.

than 2500 grams, were in the first week of life and were progressing satisfactorily. The newborn infants were selected at random from the nursery and are representative of children during the first 7 days of life. It will be seen that newborn infants possess less than half the adult concentration of carbonic anhydrase in their blood and that premature infants possess about one quarter of the adult value. These differences are statistically significant.

Table II demonstrates that large, spontaneous changes in the level of enzyme in the blood do not usually occur within a period of several days.

<sup>1</sup> Aided by a grant from the Clinical Research and Teaching Fund of the Yale University School of Medicine.



TABLE II

*Constancy of blood level of carbonic anhydrase over a period of days*

(a) *Levels obtained on newborn infants*

E per c. mm.	Interval	E per c. mm.
1.20	4 days	1.02
1.30	11 days	1.14
0.40	11 days	0.45
0.10	11 days	0.00
0.76	10 days	0.00
1.87	10 days	1.50
1.70	3 days	1.82
0.53	3 days	0.55

(b) *Levels obtained on premature infants*

Initial value	Intervals in days after initial reading with enzyme value
<i>E per c. mm.</i>	
0.28	3 days: 0.27— 5 days: 0.29
0.00	1 day: 0.00
0.40	23 days: 0.45—33 days: 0.63
0.10	11 days: 0.00
0.50	6 days: 0.45
0.75	39 days: 0.85
0.33	28 days: 0.55—45 days: 0.37
0.53	1 day: 0.50— 3 days: 0.54
0.40	1 day: 0.40
0.40	18 days: 0.51—20 days: 0.43—27 days: 0.48
0.31	20 days: 0.76
0.54	20 days: 0.43
0.93	7 days: 1.17
0.76	7 days: 0.89
0.41	7 days: 0.50
0.58	9 days: 0.51

The small variations probably represent errors inherent in the sampling and the method. The only full-term infant showing a gross change (from 0.76 to 0.00 E per c. mm.) was carefully checked and the second value was confirmed. This inexplicably low finding in an infant who was clinically quite well at the time is at variance with the data to be given below. In the case of

TABLE III

*Rise in blood level of carbonic anhydrase following transfusion (20 cc. per kilogram)*

Before blood	Interval	After blood
<i>E per c. mm.</i>		<i>E per c. mm.</i>
0.00	4 days	1.0
0.82	9 days	1.37
0.55	1 day	0.92
0.85	2 days	1.77
0.66	5 days	1.67
0.43	10 days	0.74
0.27	1 day	1.52
0.40	1 day	1.92 (2 transfusions)
0.37	1 day	2.35
0.51	2 days	1.60
1.02	1 day	1.60
0.63	2 days	1.92
0.15	2 days	2.05 (2 transfusions)
0.23	1 day	1.02
0.34	1 day	0.93

the only premature infant showing a gross change (from 0.31 to 0.76 E per c. mm.), a period of 20 days elapsed between the 2 enzyme determinations.

Table III demonstrates that transfusions of adult blood raise the level of carbonic anhydrase when the initial value is low. The data were obtained from both well and sick newborn infants. The blood used for transfusion was either drawn freshly or taken from the hospital blood bank, but the blood was never more than 1 week old. Twenty cc. per kilogram were given intravenously by the indirect, citrate method. While there is no precise correlation, it will be seen that the increase approximates the expected value when it is remembered that the average adult blood level of enzyme is 3.39 E per c. mm. and that 20 cc. per kilogram of adult blood are added to an infant blood volume of approximately 60 cc. per kilogram.

Table IV presents data which show that plasma infusions do not raise the carbonic anhydrase level of the recipient's blood. Such a rise is not to be expected, since it will be recalled that the enzyme

TABLE IV

*No rise in blood level of carbonic anhydrase following plasma infusion*

Before plasma	Interval	After plasma
<i>E per c. mm.</i>		<i>E per c. mm.</i>
0.76	7 days	0.89
0.00	1 day	0.00
0.56	5 days	0.37

is present within the red blood cells (3) and is inhibited by serum (5).

The plasma was a pooled mixture of plasma from several bloods, obtained from the blood bank, and was given in amounts of 20 cc. per kilogram of body weight. The age of the plasma presumably should make no difference in the results, since we have observed that whole blood, on standing, loses no measurable amount of its carbonic anhydrase content over a period of several days, and Lambie (6) has shown that laked blood may be stored for 5 days without loss in its carbonic anhydrase potency.

Table V shows that the rise in carbonic anhydrase content of the recipient's blood is not a transitory phenomenon and probably is sustained as long as the donor's red blood cells remain in-

TABLE V  
Sustained rise in blood level of carbonic anhydrase following transfusion

Initial value	Intervals after transfusion with enzyme value	
E per c. mm.	E per c. mm.	E per c. mm.
0.00	2 days: 1.00—	3 days: 1.40
0.40	1 day: 1.92—	10 days: 1.52
0.37	1 day: 2.35—	3 days: 1.80—16 days: 1.43
0.85	2 days: 1.77—	9 days: 1.77

tact in their new environment. Not shown in the table is the only patient whose enzyme level, raised by 2 blood transfusions from 0.12 E per c. mm. to 2.05 E per c. mm., declined to 0.43 E per c. mm. within the next 2 days. This fall was coextant with an increase in cyanosis and dyspnea and the development of jaundice. The baby died just after the last enzyme determination and autopsy showed extensive kernicterus. Unfortunately, the blood typing and cross-matching were not checked but it was felt that this infant suffered from a transfusion reaction, with hemolysis of the recipient's cells and resultant loss of carbonic anhydrase complement.

Table VI shows normal carbonic anhydrase levels obtained from infants exhibiting cyanosis. In each case, the cyanosis is adequately explained by an accompanying morbid condition. Since it will be shown below that certain types of cyanosis of the newborn are associated with a low blood level of carbonic anhydrase, it seems advisable to call attention to the fact that Table VI shows that cyanosis, *per se*, does not lower the enzyme value. This is in agreement with Hodgson (7) who found increased enzyme concentration in cyanotic adults whose blood showed high hema-

tocrit readings (congenital heart disease, bronchitis, cardiac failure).

Table VII shows that morphine is without effect on the enzyme potency of the patient's blood. It was thought desirable to demonstrate this, since the mothers of some of the babies discussed below had been given morphine at variable intervals before delivery. Furthermore, it was found that

TABLE VII  
Failure of morphine to inhibit carbonic anhydrase

Before	After
E per c. mm.	E per c. mm.
3.57	3.57
3.47	3.26
3.26	3.52
3.00	3.26

Carbonic anhydrase determinations on 4 adults before and one-half hour after gr.  $\frac{1}{4}$  of morphine sulfate.

morphine sulfate added to blood *in vitro*, in concentrations equal to a 10 grain dose given by hypodermic injection, did not significantly reduce the enzyme activity of the blood.

Table VIII presents certain clinical data in connection with the studies on carbonic anhydrase. The cases were selected because they showed cyanosis and were doing poorly at a time when no recognized explanations for these findings were discovered. Two of the infants were full-term while 11 were premature. Attention is directed to the fact that when cyanosis and failure to thrive was found unassociated with recognized cause, transfusion of adult blood led to clinical improvement. Usually the initial level of the enzyme was below 0.5 E per c. mm. but this was not invariably

TABLE VI  
Cyanosis from known cause, accompanied by normal blood levels of carbonic anhydrase

Weight grams	E per c. mm.	Clinical note
9225	2.65	
3200	1.67	Markedly cyanotic; diagnosis: congenital heart disease.
3600	1.67	Markedly cyanotic; difficult resuscitation; bulging fontanel; bloody cerebrospinal fluid; died after 3 days; no autopsy; diagnosis: probable intracranial hemorrhage.
1655	1.02	Severe cyanotic spells; diagnosis: low calcium tetany (blood calcium: 6.4 mgm. per cent).
1600	1.43	Markedly cyanotic; died after 2 days; autopsy: tracheo-esophageal fistula.
		Markedly cyanotic; died after 1 day; autopsy: intracranial and intraperitoneal hemorrhage.

TABLE VIII  
*Infants with unexplained cyanosis: their subsequent course*

Weight	1st day of life	Clinical note
<i>grams</i>	<i>E per c. mm.</i>	
1. 2935	0.73	Very cyanotic; died in 4 hours; no cause apparent; no autopsy.
2. 2770	0.85	Seemed well but persistently cyanotic without demonstrable cause; at 4 days, transfused, E: 1.77; cyanosis cleared within 24 hours of transfusion; at 5 days, E: 1.77; continued to do well.
3. 1040	0.38	Very cyanotic and feeble; died in 2 hours; complete autopsy showed only minimal atelectasis.
4. 2490	0.26	Cesarean section; mother had gr. $\frac{1}{4}$ morphine 1 hour before delivery; infant feeble, cyanotic; no apparent cause; died in 3 hours; no autopsy.
5. 2330	0.28	Cyanotic for first 3 days of life without apparent cause; enzyme level unchanged at the end of this period (E: 0.27); the following day, color good and E: 0.43 (infant had not been transfused); continued to thrive.
6. 1110	0.00	Cyanotic and feeble without apparent cause for 3 days; after plasma infusion, no improvement and E: 0.00; on fourth day of life, transfused; immediate disappearance of cyanosis and E: 1.0; did well thereafter.
7. 1030	0.27	Very cyanotic and seemed moribund; transfused at 2 days and seemed better immediately with E: 1.52; died suddenly next day; autopsy: subtentorial hemorrhage.
8. 1300	0.40	Color only fair; eating and gaining poorly; transfused with immediate improvement in appetite, color, and weight gain which was sustained thereafter; E: 1.92.
9. 1270	0.12	Cyanotic, sucking poorly, minimal atelectasis; after 2 transfusions, E: 2.05 but baby did poorly, became increasingly cyanotic and died after 2 days, with E: 0.43 just before death; autopsy: marked kernikterus; question of transfusion reaction.
10. 745	0.23	Very feeble, weak cry, poor respirations, cyanotic even in oxygen; after 1 day and transfusion, E: 0.93; immediate sustained improvement in general status; after 4 days, E: 1.18; after 11 days, E: 1.17; continued to thrive.
11. 905	0.34	Very low vitality, weak cry, cyanotic, apneic periods; after 1 day and transfusion, E: 1.02; seemed much stronger, sucked well, color better, more vigorous cry; after 4 days, doing well, out of oxygen, E: 1.07; continued to thrive.
12. 1405	0.43	Severe spells of cyanosis, poor appetite, low vitality; immediate, sustained improvement after transfusion and E: 0.74.
13. 1775	0.82	Seemed well but persistently cyanotic until transfused on fifth day; immediate disappearance of cyanosis; E: 1.37; continued improvement thereafter.

the case. In the 2 full-term infants (cases 1 and 2), although the levels are almost 2 standard deviations below the average newborn level, the initial values are not low by premature standards. One of these patients died without a transfusion or autopsy so no conclusion can be made except that the enzyme was somewhat low. In the other case, transfusion led to striking and prompt clinical improvement and increase in the level of enzyme.

Of the premature infants, 2 (cases 3 and 4) died before transfusions could be given. In one

case, autopsy failed to reveal adequate anatomical cause for cyanosis while in the other case, autopsy was not permitted. In this case, morphine poisoning was suspected but, if present, it cannot explain the low level of carbonic anhydrase since it has been shown that morphine is without measurable effect on the activity of carbonic anhydrase (Table VII). In the case of number 5, the cyanosis disappeared at the same time that a spontaneous rise in the blood level of the enzyme developed. The case probably would not have been included here except for the fact that it was one of the group

born infants who exhibited unexplained cyanosis. Even though the increase in carbonic anhydrase is minimal, the correlation is suggestive. Initial rate of a normal enzyme level, number 13 continued to exhibit cyanosis until transfusion almost doubled the carbonic anhydrase value. The remaining infants all showed a great clinical improvement after their low levels of enzyme had been raised by transfusion of whole blood. Following a transfusion, number 7 improved from a moribund condition to such an extent that recovery was anticipated. The sudden death, due to subdural hemorrhage, is felt to represent an additional complication. Number 9 has been commented upon previously (Table V); it is felt that the failure to sustain the rise in enzyme concentration and the poor course following transfusion, when considered with the autopsy finding of kernicterus, suggest a transfusion reaction with hemolysis of the donor's red cells. Although Rh studies were not carried out in this case, it was felt that erythroblastosis fetalis could be excluded by the absence of clinical or pathological evidences of this disease.

While the transfusions undoubtedly had other effects than raising the level of the carbonic anhy-

draz, none of the usual indications for transfusion was present. None of the infants was dehydrated or exhibited good evidences of shock. There were no clinical evidences of anemia and the hemoglobins were demonstrated to be over 15 grams in those cases on which initial values were estimated. No reactions occurred except as noted in case 9.

In Figure 1, the levels of carbonic anhydrase are plotted against birth weight. The babies showing unexplained cyanosis are designated by crosses and those with cyanosis from recognized causes are marked by circles. It will be seen that low levels of carbonic anhydrase are found in newborn infants of all weights including occasional full-term infants (not shown in the Figure). However, none of the 10 infants weighing less than 1500 grams at birth showed levels of carbonic anhydrase greater than 0.5 units. Thus, a certain degree of maturity is necessary for the development of high levels of carbonic anhydrase, but occasionally, the enzyme fails to develop in otherwise mature, newborn infants.

The figure also shows that, with one exception, the instances of unexplained cyanosis occur in infants with levels of carbonic anhydrase below 0.5

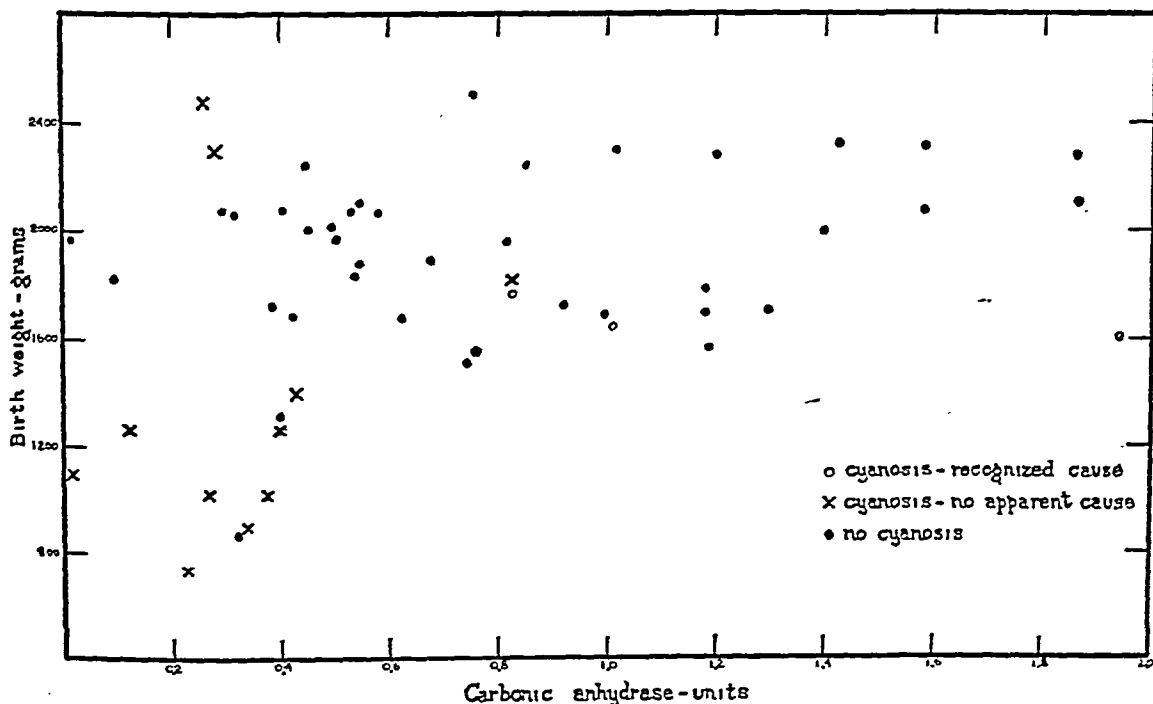


FIG. 1. THE RELATION BETWEEN BIRTH WEIGHT AND ENZYME LEVEL IN THE BLOOD

units. However, as was pointed out in connection with Table VIII, 2 full-term infants showed unexplained cyanosis with levels of carbonic anhydrase of about 0.8 units. Of the 21 infants with levels below 0.5 units, 10 showed cyanosis. Of these 10, with unexplained cyanosis, 8 weighed less than 1600 grams.

Since the Figure shows very low levels in thriving infants, low levels of carbonic anhydrase do not directly lead to cyanosis nor necessarily interfere with apparent well-being.

#### DISCUSSION

Although no precise relationship between birth weight and the level of carbonic anhydrase can be demonstrated, small premature infants (1500 grams or less) tend to show the lowest levels of enzyme. It will be recalled that Meldrum and Roughton (2) demonstrated that the level of enzyme in goat fetuses does not begin to rise until very near term. For this reason the level of carbonic anhydrase may be considered one of the measures of maturity in the newborn. Hall (8) has suggested that there exists a fetal form of hemoglobin, with a greater than normal affinity for oxygen, which is adapted to intrauterine existence with its low oxygen tension. It is possible that the low level of carbonic anhydrase in premature infants is a characteristic of this fetal hemoglobin.

Roughton (9) has demonstrated mathematically that conversion of bicarbonate to carbon dioxide without carbonic anhydrase would be so slow as to be the limiting factor in respiratory exchange. However, he has also shown that the levels of enzyme in adults accelerate the reaction 100 times as much as is necessary, but this margin of safety decreases when maximum activity is undertaken. Lambie (6) has shown that the blood level of the enzyme can be reduced to 22 per cent of the normal without demonstrable ill effects in adults. Since there is this margin of safety and newborn infants are relatively inactive, the level of carbonic anhydrase in premature and full-term newborn infants is theoretically and practically adequate. This does not mean that difficulties due to low carbonic anhydrase may not develop in active babies. Indeed, the frequency of cyanotic spells in premature infants after feeding and exertion or during infections suggests that such is the case.

However, it must be recalled that one baby showed no symptoms in spite of a blood carbonic anhydrase level of 0.00. While measurements of pH and carbon dioxide tension would be necessary to prove that there were no physiological disturbances due to the low carbonic anhydrase, the observation proves that very low levels are compatible with life in a premature infant.

There are theoretical reasons why a low blood level of carbonic anhydrase might interfere with oxygenation of the blood. Oxygenation of hemoglobin is physiologically speeded when the blood gives up carbon dioxide in the lungs (10). Also, the hemoglobin dissociation curve is shifted to the left in proportion to the lowering of carbon dioxide tension. It is possible that a low blood level of carbonic anhydrase delays the lowering of the carbon dioxide tension in the lung capillaries, resulting in a slow and incomplete oxygenation of hemoglobin. In this connection, it is interesting to note that Smith and Kaplan (11) have shown that the arterial blood of premature infants generally possesses a lower oxygen saturation than that of adults or full-term infants of comparable age.

It is well known that patients being treated with sulfanilamide are prone to develop cyanosis and it is interesting to note that sulfanilamide (alone, of all the sulfonamides) inhibits the action of carbonic anhydrase (12, 13). We realize that this neglects the observations that the cyanosis of sulfanilamide therapy may be due to methemoglobin (14, 15).

The poor appetite of many of the infants with low carbonic anhydrase quite possibly may be related to a lessened secretion of gastric juice. Davenport (16) has shown that the adequate function of the parietal cells of the gastric mucosa is dependent on the presence of carbonic anhydrase and it has been suggested previously (13) that the anorexia accompanying sulfanilamide therapy may be related to inhibition of the enzyme.

#### SUMMARY AND CONCLUSIONS

Because Meldrum and Roughton (2) had found goat fetuses low in carbonic anhydrase, because carbonic anhydrase is found with hemoglobin in the red blood cells (3), because of a clinical impression that some premature and full-term new-

born infants, who were doing poorly and who were exhibiting cyanosis, improved after whole blood transfusions, an attempt was made to correlate unexplained cyanosis in newborn infants with low concentrations of carbonic anhydrase in the blood.

The concentration of carbonic anhydrase in the blood of newborn infants is less than one-half and the concentration in premature infants is only one quarter of that found in the blood of adults. Spontaneous changes in the blood enzyme level do not usually occur but significant increase in the level can be accomplished by whole blood transfusions.

Thirteen infants were studied who exhibited cyanosis, unexplained by recognized physical causes, and who were doing poorly. In many instances, these infants showed levels of carbonic anhydrase which were significantly low. Improvement in respect to cyanosis and general condition was accompanied by a rise in the blood concentration of carbonic anhydrase. The rise occurred in one infant spontaneously and was minimal. In the remaining infants, the rise followed transfusion and was significant in most instances.

The findings show that many premature infants who are doing poorly and exhibit cyanosis have low levels of carbonic anhydrase. Following transfusions of adult blood, the level of carbonic anhydrase increases and clinical improvement, accompanied by a disappearance of cyanosis, follows.

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GROUP A HEMOLYTIC STREPTOCOCCUS ANTIBODIES. I.  
GRIFFITH TYPE AGGLUTININ AND ANTISTREPTOLYSIN  
TITERS IN NORMAL MEN AND IN  
ACUTE INFECTIONS

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The immunological phenomena associated with various aspects of hemolytic streptococcus disease in man have usually been studied by the measurement of antitoxins. Most extensive have been the observations made by the Dick technique (1), in which purified erythrogenic toxin was used as a skin test material. Circulating antistreptolysin and antifibrinolysin titers have also been determined, particularly in individuals suffering from glomerulonephritis and rheumatic fever (2).

The measurement of antibacterial antibodies has been less satisfactory. Phagocytosis (3) and the destruction of the living organism by whole blood (4) have been the most recent methods of choice.

The agglutination of hemolytic streptococci by human serum has also been frequently studied (5). Many attempts were made during the first two decades of this century to establish the etiological relationship between streptococci and scarlet fever and many other diseases by the application of agglutination tests, using as antigens streptococci isolated from various sources. Usually, it was demonstrated that the convalescent serum of individuals suffering from infectious processes, now known to be caused by the hemolytic streptococcus, had higher titers of agglutinins than had controls, but the difficulties inherent in the production of stable antigens and the many confusing cross reactions caused a nearly complete abandonment of the phenomenon of agglutination as a means of study of streptococcus disease.

Much more recently several studies have been made of the hemolytic streptococcus agglutinin content of the sera of individuals suffering from various forms of arthritis, which have indicated that increased amounts of these antibodies are present in association with the rheumatoid type (6). The variations from the normal have not

been sufficiently great to permit the use of the agglutination test in the diagnosis of disease of the joints, nor to establish definitely the role of the streptococcus in the causation of rheumatoid arthritis.

One group has described the agglutination of hemolytic streptococci by high dilutions of serum obtained from febrile patients known not to be suffering from streptococcus infections (7).

It has been recently demonstrated that nearly all serious human infections, particularly those involving the respiratory passages, are caused by hemolytic streptococci serologically homogenous, the members of the Lancefield Group A (8). By the application of a technique of slide agglutination, more than 20 types have been identified within this group (9). Antibacterial immunity to infection with organisms of Group A, like that of the pneumococci, has been shown to be largely type specific (10). It is apparent, therefore, that the satisfactory study of the agglutinin content of human sera for the hemolytic streptococci pathogenic for man must be conducted in such a way that antigens made from strains of many types may be used. This can easily be accomplished by the application of a slide technique, first described by Ozaki (11), who, using antigens of all of the Griffith types, found that each was agglutinated by a small number of normal sera. Later, other Japanese workers (12) studied acute and convalescent sera obtained from individuals ill with scarlet fever. They also demonstrated that agglutinins for many types were present and that 39 per cent showed a definite increase in the amount of antibody for the homologous type of streptococcus during convalescence. Walker (13) also studied the development of agglutinins in scarlet fever by the slide method and showed that these antibodies appeared in approximately 25 per cent of all cases and that they were type specific.



In this study, the agglutinins present in normal sera have been measured by a slide technique, as has the agglutinin response to acute infection with the hemolytic streptococcus. Both have been compared with antitoxic immunity by means of antistreptolysin determination.

## MATERIALS AND METHODS

### *Sera*

All bloods were collected by venipuncture and the sera removed from the clots in the usual manner and stored at 5° C. in the cold room. All sera were inactivated at 56° C. for 30 minutes and the antistreptolysin titers determined before the agglutination tests were performed.

*a. Preparation of antigens.* Antigens for the slide agglutination tests were prepared and the tests performed in the same manner as has been previously described (14), using standard type specific strains of Group A hemolytic streptococci, obtained from the Lederle Laboratories. Many sera were tested against antigens of Types 1, 2, 4, 6, 9, 11, 12, 13, and 25.

Frequently an antigen was prepared from an organism isolated from the individual under study and used in the tests. It proved to be much more satisfactory to use antigens of the same type, prepared from stock strains which were known to give smooth, stable suspensions if the homologous organism could be identified serologically. Satisfactory antigens could be prepared only if the organism was grown very slowly. Incubation at ordinary room temperatures in a rotating box usually gave excellent conditions for this purpose, but it was noted that marked granularity and instability developed in antigens incubated on very warm days.

If these precautions failed to induce the development of sufficiently smooth suspensions, a drop of 10 per cent trypsin solution was added to the thick agglutinating suspension and the whole was incubated at 37° C. for approximately 15 minutes. This procedure was invariably followed by the development of a stable but somewhat insensitive antigen. It should be emphasized that suspensions of streptococci quite satisfactory for Griffith typing may well be too unstable for use in the testing of human sera and false positive reactions will be obtained.

A stable, diffuse antigen having been obtained, it was found to remain in a satisfactory state for many months if the living culture was stored at 5° C. in the refrigerator when not in use. No preservative was added. Each time tests were to be made the antigens were checked with known negative and positive sera. The latter were diluted to three-fourths of their agglutinin titer. This precaution was especially important if any of the antigens contained trypsin.

*b. Preparation of sera.* The heated human sera were first tested against the various Group A type specific antigens by means of slide agglutination tests in the undiluted state. Further tests were then performed with saline di-

luted serum against those types showing clumping in one minute or less.

All agglutinin titers in this and the following report are described in terms of the actual dilution of the serum used in the tests. In the slide test, a small drop of serum was mixed with a large drop of antigen suspension, so the actual dilution titers were, therefore, from 2 to 4 times greater than those presented in these reports.

### *Antistreptolysin titers*

The antistreptolysin titer of each serum was determined by the method described by Coburn and Pauli (15), who were kind enough to supply lyophilized cultures of the Aronson strain of streptococcus and generous amounts of standard human sera.

### *Throat culture*

Swabs were carefully rubbed over the posterior pharynx and in both tonsillar fossae. Cultures were prepared and serological classification of the isolated hemolytic streptococci was performed using the techniques previously described (14, 16).

## PROCEDURE AND RESULTS

### *Normal individuals*

The sera of 47 normal, healthy, male, medical students were studied to determine the presence of agglutinins for Group A streptococci of Types 1, 2, 4, 6, 9, 11, 12, 13, and 25, which were responsible for 60 to 75 per cent of all instances of infection in the San Francisco area during the two previous years. The antistreptolysin titer of each serum was also measured.

Throat cultures were obtained from each of the studied individuals, all of whom had undergone previous tonsillectomy more than one year previously. None harbored Group A hemolytic streptococci in the naso-pharynx. The results of study of carriers of these organisms will be presented in the following paper.

*Results.* Figure 1 summarizes the serological observations on these 47 normal subjects. The highest agglutinin titer for any of the 9 types of streptococcus obtained with each serum and the antistreptolysin titer was used to construct the figure. Of the sera, 53.2 per cent contained no agglutinins for the studied Group A hemolytic streptococci and 21.2 per cent had demonstrable agglutinins only when the undiluted serum was used in the tests. The agglutinin titer was 1:4, or more, in 25.6 per cent.

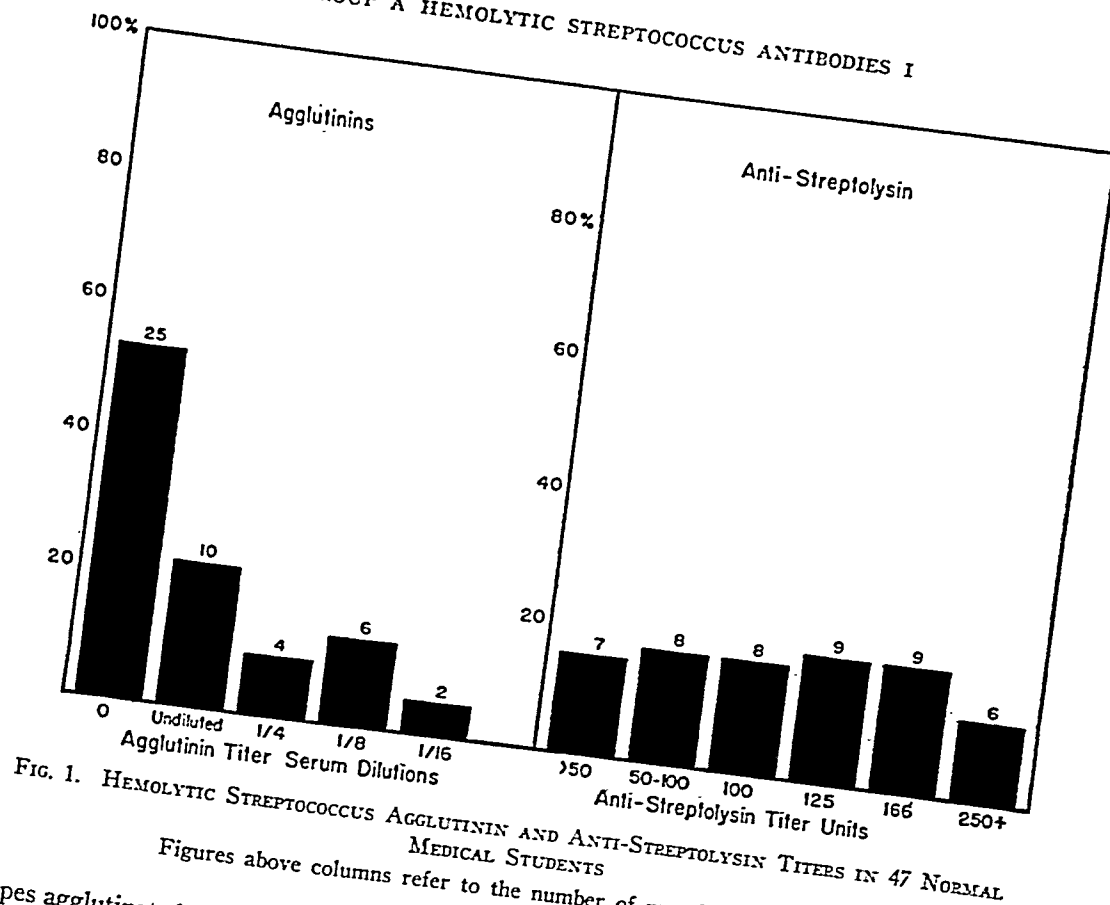


FIG. 1. HEMOLYTIC STREPTOCOCCUS AGGLUTININ AND ANTI-STREPTOLYSIN TITERS IN 47 NORMAL MEDICAL STUDENTS  
 Figures above columns refer to the number of men in each group.

The types agglutinated and the titers of the sera for each type are presented in Table I. Twenty-two positive sera agglutinated 33 antigens, since some sera caused the agglutination of more than one type. Similar cross reactions will be discussed later. Agglutinins for Types 1, 9, 12, and 13 were not discovered in any serum, and antibodies for Types 2, 6, and 25 were most frequently present. The antistreptolysin titers of 31.9 per cent of the serums obtained from these normal individuals were greater than 125 units, but only 12.7 per cent were above 166 units.

TABLE I  
 Types of group A streptococci agglutinated by the sera of normal medical students

Titers	Griffith types								
	1	2	4	6	9	11	12	13	25
Undiluted		5	3	5		1			3
1:4		2	1	2		1			1
1:8		3				1			2
1:16		1		1					1
Totals		11	4	8		3			7

Very careful histories were obtained from these subjects in relation to previous infections with the hemolytic streptococcus, such as scarlet fever and/or repeated attacks of tonsillitis. In 24 instances, it was probable that such infections had not occurred, whereas in 15, a definite past history of streptococcus disease was obtained. Four were doubtful and 4 were arbitrarily placed in the previously infected group because, at the time of this study, the antistreptolysin titers of the sera of these men were greater than 250 units.

A study of the agglutinin titers in relation to this division of cases is of interest. Only 8 per cent of the sera obtained from individuals with a negative past history had agglutinin titers of 1:4 or more, and two-thirds contained no agglutinins at all. Of those with a past history of hemolytic streptococcus infection, or who had a markedly elevated antistreptolysin level, agglutinin titers of 1:4 or more were found in 47.5 per cent and only 42 per cent had no agglutinins.

A scatter graph was constructed to determine the relation of the agglutinin to antistreptolysin

titers but has not been presented since no definite correlation between the two values was discovered.

Acute infections

The agglutinin and antistreptolysin response during acute infection by the Group A hemolytic streptococcus was determined by the frequent measurement of these antibodies during the course of 24 cases of typical scarlet fever, studied from the first through the third or fourth week of the disease. Twenty-two of the infected individuals were children under the age of 14 years, and 2 were adults. Only one suffered a suppurative complication in the form of an otitis media. Fifteen received full doses of a sulfonamide early in the course of the disease.

*Agglutinin response.* Agglutinins for the homologous type of streptococcus developed in 13 of the 24 cases in whom these antibodies were not present at the onset of the disease. In two cases, the serum contained type specific agglutinins on the fourth and sixth days, respectively. In one

TABLE II  
The relation of the Griffith type of infecting streptococcus to the maximum agglutinin titer for the homologous type in 22 cases of scarlet fever

Maximum agglutinin titer	Griffith types								Total
	2	3	4	6	11	23	25	Unidentified	
Negative			1	1	5			2	9
Undiluted					1				1
1:4		1		1					2
1:8	1						1	1	3
1:16				2	2		1		5
1:32							2		2

of these, the dilution titer was greater than 1:16, in the other, it was not measured.

When an agglutinin response occurred, the level of circulating antibody usually increased sharply during the sixteenth to twentieth day after the onset of the infection and at a time when convalescence was well established. In two instances, elevated titers appeared on about the twelfth day. These relationships are summarized in Figure 2.

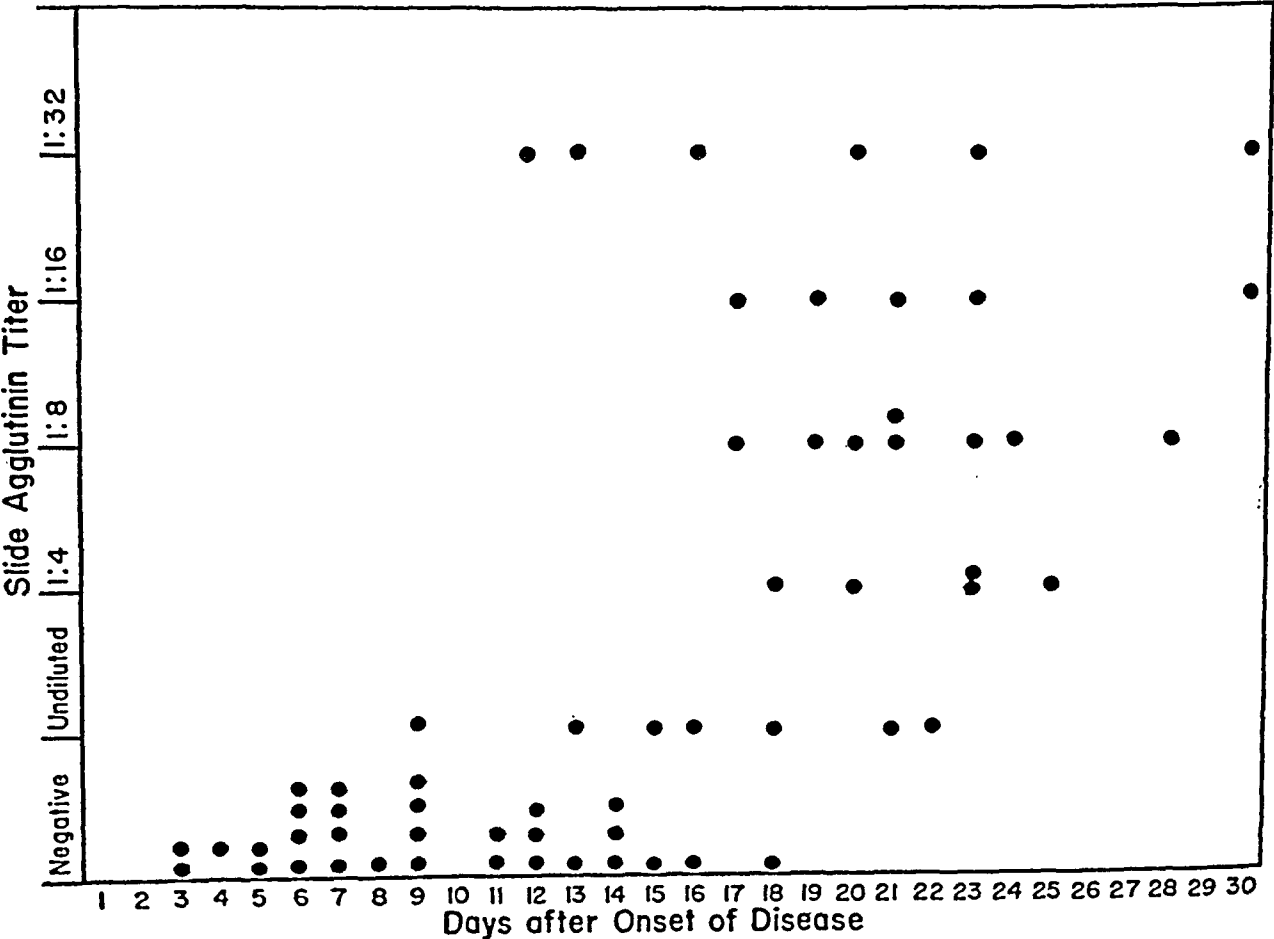


FIG. 2. AGGLUTININ RESPONSE IN 13 SCARLET FEVER PATIENTS

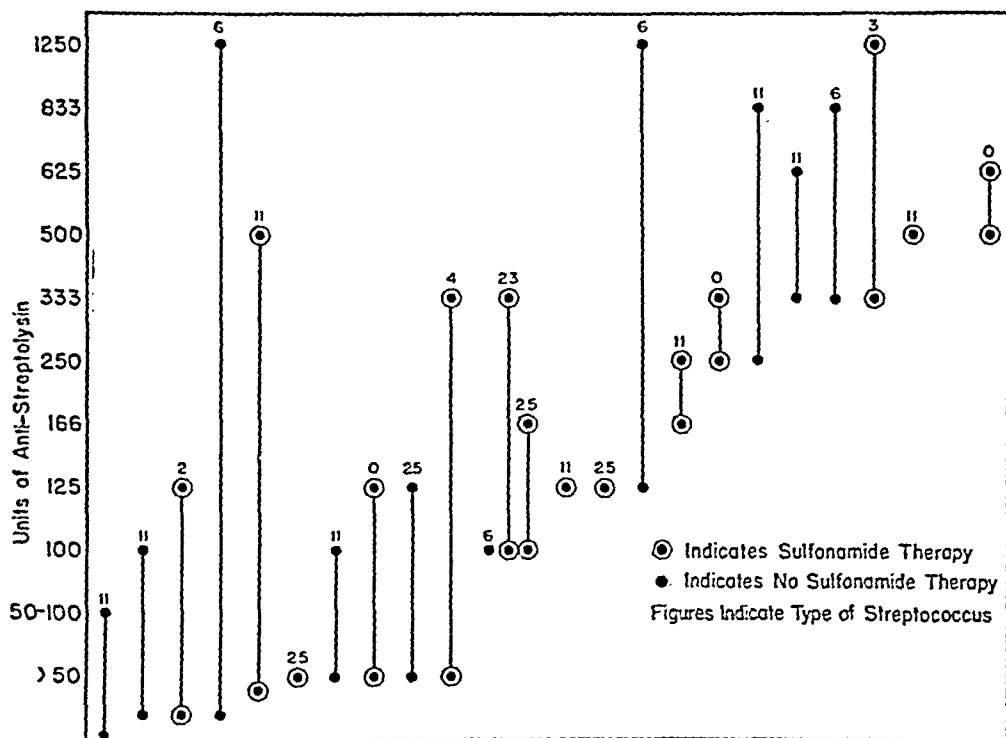


FIG. 3. MAXIMUM AND MINIMUM ANTISTREPTOLYSIN TITERS IN 24 CASES OF SCARLET FEVER

In Table II, the maximum agglutinin titer for the homologous organism is compared with the type of infecting streptococcus.

A cursory examination of these data indicates that Type 11 failed to stimulate an agglutinin response much more frequently than the other types. A more critical analysis reveals that this effect may have been due to the use of sulfonamides in the treatment of certain of these cases. Thus, only 6 of 13, or 46.2 per cent, of drug-treated cases developed agglutinins, where 7 of 9, or 77.8 per cent, of those not so treated did so. Furthermore, response occurred in 3 of 4 untreated Type 11 cases, but none was demonstrated in 4 who received a sulfonamide. Insufficient numbers of cases of the other types were observed to draw further conclusions as to the relationship between chemotherapy and the development of these antibodies.

*Inter-type cross agglutination.* In 9 instances, sera were examined for cross agglutination with other than the homologous organism, using antigens prepared from the 8 types previously used in the study of normal sera. In the following paper, the presence of high titers of agglutinins for Group

A streptococci in the sera of tonsillar carriers of these organisms will be described. Observations were made in these 33 cases for the presence of cross agglutination reactions as well. The results of the study of these 42 cases may well be presented at this time.

In 11 instances, no agglutinins for the homologous type were demonstrable. Antibodies were discovered in 6 of these, in titers greater than 1:4, for one or more of the other types. Cross agglutinins were not demonstrated in 9 of those individuals in whom homologous agglutinins were definitely present. Among the others, the heterologous agglutinin titer roughly paralleled the homologous, but was often higher.

The most frequent cross reactions were Type 6 with Type 2; Type 11 with Types 1 and 6; Type 12 with Types 6 and 2; and Type 25 with Type 2. It is interesting that in instances of infection with Types 11 and 12, cross agglutinins for Type 6 usually were present, but when Type 6 was the infecting organism, agglutinins for the other two types were never found.

*Antistreptolysin response.* An antistreptolysin response to infection with the Group A strep-

tococcus occurred in 19 of the 24 cases of scarlet fever that were studied. The increase in level of circulating antitoxin usually accompanied or preceded the agglutinin response and developed on the thirteenth to eighteenth days of the infection.

Four of the 5 patients in whom no antistreptolysin response occurred were treated with sulfonamides. It is of interest to note that the 5 individuals who did not form antistreptolysin also failed to develop agglutinins. In Figure 3, the initial and maximum antitoxin titers are presented. In the first week, they were greater than 250 units in 6 instances. It should also be noted that patients with low initial titers tend to have low maximum titers and the reverse, but that there are notable exceptions to this generalization. Correlation between the type of infecting streptococcus and the antistreptolysin response was not observed, but the 2 cases showing the most striking increase in titer were both due to organisms of Type 6.

#### DISCUSSION

These observations indicate that the measurement of hemolytic streptococcus agglutinins in human sera by means of a slide technique is a satisfactory procedure. Most important is the use of stable antigens, and methods for their preparation have been described.

A study of 47 normal young males, who were not carriers of Group A streptococci, using the antigens prepared from those types of these organisms most frequently the cause of disease in the San Francisco area, showed that approximately 47 per cent contained agglutinins for one or more types. In only 25 per cent, however, were the titers as great as 1:4. Such levels were present in only 8 per cent of individuals with no past history of hemolytic streptococcus disease, but were found in 47 per cent of those who had experienced infection by this organism. In the latter instance, the agglutinin titer was rarely greater than 1:8. This evidence suggests that these antibodies may be maintained for a long time after the recovery from the responsible disease, since none of the studied group admitted a recent infection. The range of antistreptolysin titers in this group was approximately the same as that reported in other centers. No correlation was

demonstrated between the amount of circulating agglutinin and antistreptolysin.

Twenty-four cases of scarlet fever were studied to determine the nature of the antibody response following acute infection by the Group A hemolytic streptococcus. An agglutinin response was found to occur in approximately one-half of these individuals, and usually accompanied or followed the rise in antistreptolysin titer during the sixteenth to twentieth days of the disease. Agglutinins for other types of streptococcus also appeared in several instances.

Sulfonamide medication appeared to interfere with the agglutinin response but the number of cases studied is too small for definite conclusions to be drawn.

The sera of two individuals agglutinated the infecting streptococcus at the onset of the disease. In one, the antibody was studied. It was present in high titer and was type specific. This observation indicates that, while the presence of type specific agglutinins for the hemolytic streptococcus may be an indication of the presence in the individual of antibacterial immunity, the presence of these antibodies is not sufficient to guarantee resistance to infection. Approximately 20 per cent of the total group failed to develop an increase in titer of antistreptolysin and no agglutinin response occurred in these individuals, only one of whom was not a sulfonamide-treated case.

The application of the slide agglutination test to other disease, using antigens of several types of Group A hemolytic streptococci, is in progress. It will be of interest to determine whether agglutinins are present in the sera of individuals suffering from rheumatoid arthritis and rheumatic fever, but it seems doubtful that this technique will be applicable to the routine diagnosis of any disease, since increased amounts of these antibodies are often present in the sera of normal individuals.

#### SUMMARY

1. Agglutinins for Group A hemolytic streptococci of various Griffith types may be demonstrated in human serum by a slide technique.

2. Such agglutinins are present in the sera of well persons and are discovered much more frequently and in higher titer in those with a past history of hemolytic streptococcus infection.

3. An agglutinin response to the infecting type of hemolytic streptococcus, and often to other types as well, occurred in approximately 50 per cent of 24 cases of scarlet fever.

4. Suggestive evidence is presented that sulfonamide medication occasionally interfered with the development of agglutinins and antistreptolysin.

5. In 2 cases, agglutinins for the homologous type were present at the onset of the acute infection, so their presence is not an indication of antibacterial immunity.

6. The slide agglutination test may be of value in the study of rheumatic fever, rheumatoid arthritis, and also disease of known streptococcal origin.

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# GROUP A HEMOLYTIC STREPTOCOCCUS ANTIBODIES. II. GRIFFITH TYPE AGGLUTININ AND ANTISTREPTOLYSIN TITERS IN CARRIERS AND NON-CARRIERS

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In the previous paper, hemolytic streptococcus antibacterial and antitoxic antibodies were studied by the determination of agglutinins for various Griffith types and antistreptolysin, in the sera of normal individuals who were not shown to be nasopharyngeal carriers of Group A streptococci. It is the purpose of this report to describe a more extensive study of these antibodies in carriers and non-carriers of hemolytic streptococci of Group A. This program was prompted by two previous observations.

First, and most important, were the carefully controlled studies of Bloomfield and Felty (1) who, in 1926, pointed out that individuals harboring hemolytic streptococci in the tonsils were immune to infection by these organisms until the carrier state had terminated. It therefore seemed possible that high titers of circulating antibody might be demonstrable in the sera of Group A carriers.

A further incentive to this work arose from the study of circulating antibodies in individuals suffering acute attacks of hemolytic streptococcus pharyngitis (2, 3), in whom it was observed that very great variations existed from patient to patient in the level of antistreptolysin at the onset of the infection and in the rate of fall after recovery. The high titers demonstrated in some of these individuals might have been maintained by a prolongation of the carrier state after previous infection. The study of hemolytic streptococcus carriers is, however, greatly complicated by the fact, previously emphasized (4), that ordinary throat swabs will recover the organism in a relatively small number of cases, since it inhabits the crypts and not the surface of the tonsil. This is particularly true if no recent episode of acute infection has occurred.

In the present study, sera for antibody determinations were obtained from children who were

about to undergo tonsillectomy and the entire excised tonsil was then examined by cultural methods suitable for the isolation of Group A streptococci. This procedure has permitted the precise separation of these individuals into carriers and non-carriers.

The results of the determination of the levels of circulating antibodies in this group will now be presented.

## MATERIALS AND METHODS

Sera, throat swabs, and a careful history were obtained from 64 children who were about to undergo tonsillectomy. Later, the excised tonsils were cultured.

## ANTIBODY DETERMINATION

The levels of circulating agglutinins for the various common local types of Group A streptococci and of antistreptolysin were determined in the same manner as that described in the previous report (2).

## BACTERIOLOGICAL OBSERVATIONS

Throat swabs and excised tonsils were studied culturally and the isolated hemolytic streptococci were classified serologically by means of the techniques that have been described in detail elsewhere (4, 5). Group A hemolytic streptococci were recovered from preoperative throat swabs in only 55 per cent of individuals, later proved to be carriers by culture of the excised tonsils.

## RESULTS

Of the 64 children studied, 33 were proved to be carriers of Group A hemolytic streptococci.

Because the proximity of active infection is important in the consideration of antibody mechanics, a history of recent or remote streptococcus infection was sought in each case. Thirty per cent of the carriers and 37.1 per cent of the non-carriers had suffered a sore throat within 4 weeks, and 66.6 per cent and 51.9 per cent, respectively, within 16 weeks. Two in each group had had scarlet fever. No correlation between a history



of recent infection and the level of circulating antibodies could be established.

*Agglutinins and antistreptolysin.* In Figure 1, the results of the determination of agglutinins and antistreptolysin, in relation to the presence or absence of the carrier state, are correlated graphically. Agglutinins were determined, using antigens prepared from Group A streptococci of Types 1, 2, 4, 6, 9, 11, 12, 13, and 25, and also, in carriers, the homologous type if it was not included in the previous group. In the figure, the highest agglutinin titer for any type was plotted. In 27 per cent of the carriers, this was not the homologous type. These cross reactions have been described in the previous paper.

Of group A carriers, 84.9 per cent had agglutinin titers greater than 1:8, and in only 9.1 per cent, were no agglutinins demonstrated. Among the non-carriers, the situation was entirely different. Here, titers above 1:8 were present in only 12.8 per cent of the group, and 61.5 per cent had no agglutinins for any of the studied types.

Somewhat similar but less dramatic were the results obtained by correlating antistreptolysin titers with the presence or absence of the carrier state. The figure reveals that 45.5 per cent of individuals who harbored Group A streptococci in the nasopharynx had antistreptolysin titers above

250 units and only 30.2 per cent were as low as 100 units or less. The level of antistreptolysin was above 250 units in 22.4 per cent, and 100 units or less in 61.5 per cent of the non-carriers. It is of interest to note that Group C hemolytic streptococci were isolated from the tonsils of 2, and Group G from 1 of the 7 individuals in this group whose antistreptolysin titers were greater than 250 units.

*Follow-up studies.* When it became apparent that elevated antibody titers were demonstrable in most of the carriers of Group A streptococci, it became desirable to determine the fate of these substances after termination of the carrier state by tonsillectomy. Consequently, 21 of the Group A carriers were restudied after intervals from 26 to 100 days, and the levels of agglutinins and antistreptolysin were determined. Throat cultures were obtained and in no case were Group A streptococci resident in the nasopharynx at the time of re-examination. Follow-up antistreptolysin titers are charted in Figure 2. It will be observed that definite fall in the level of this antibody occurred in 19 of the 21 individuals and that the rate and relative amount of this change was fairly constant for any initial antibody titer.

Similar results were not obtained in follow-up studies of the agglutinin titers. Satisfactory ob-

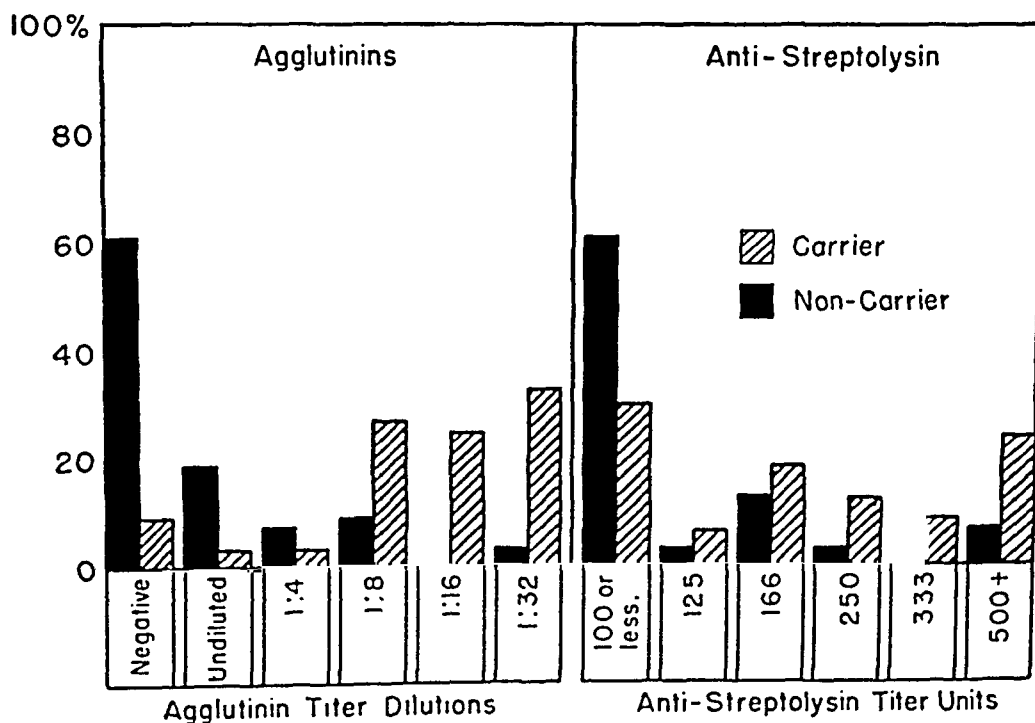


FIG. 1. AGGLUTININ AND ANTISTREPTOLYSIN TITERS IN CARRIERS AND NON-CARRIERS OF GROUP A HEMOLYTIC STREPTOCOCCI

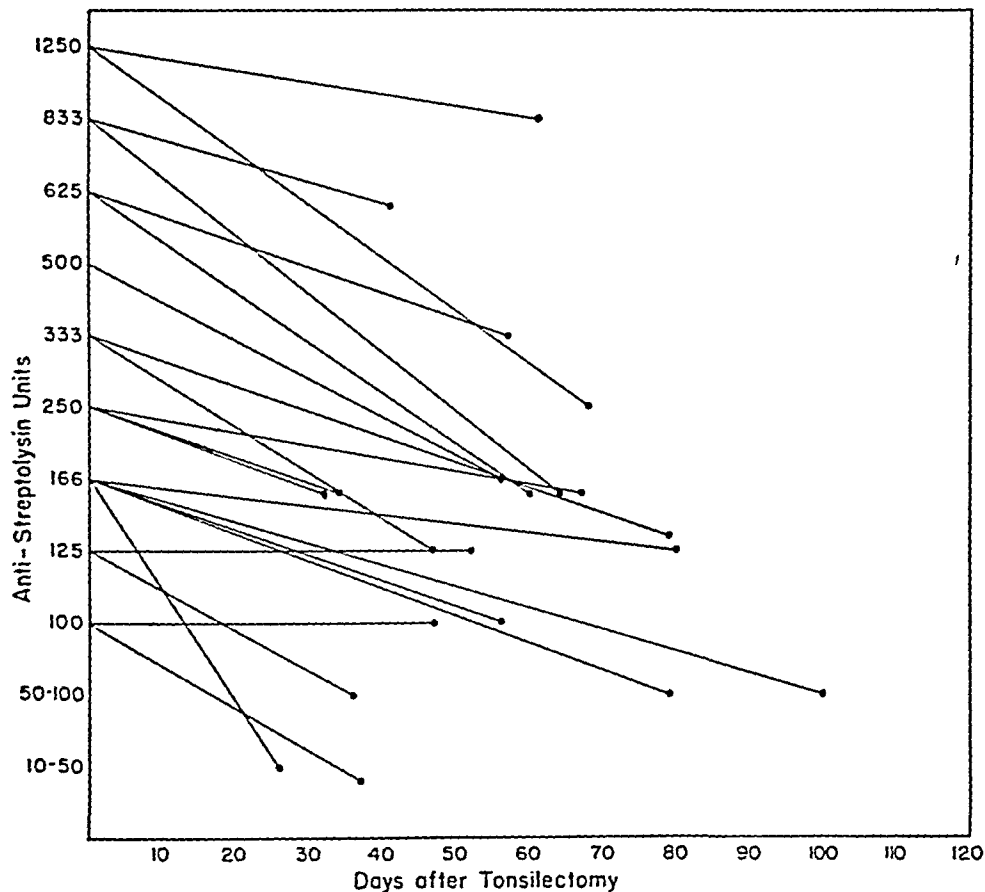


FIG. 2. FALL IN ANTISTREPTOLYSIN TITER IN 21 GROUP A HEMOLYTIC STREPTOCOCCUS CARRIERS AFTER TONSILLECTOMY

servations were available in 17 cases from 40 to 80 days after tonsillectomy. No change had occurred in the agglutinin levels of 9, 5 had fallen 1 dilution, and 3 had fallen 2 dilutions. These changes were not correlated in any way with the interval of time since operation.

*Results of Griffith typing.* The Griffith types of the isolated Group A streptococci were determined, the distribution being presented in Table I.

Types 6, 12, and 25 were most frequently isolated from the tonsils of these individuals. In 9

instances, agglutinins for the homologous type were absent from the serum of carriers. Two of these children harbored type 4; 3, type 6; and 3, unidentified types. In 6, agglutinins for other types were discovered. No other correlation between Griffith type and any antibody level was established.

#### DISCUSSION

The observations described in this paper indicate that tonsillar carriers of Group A streptococci have elevated levels of circulating antistreptococcal antibodies more frequently than do non-carriers. These differences are most easily demonstrable by the study of agglutinins, since nearly all carriers have markedly increased amounts of these antibodies and only a few non-carriers do so. A similar but less marked trend is apparent when the antistreptolysin titers are considered.

TABLE I  
Distribution of Griffith types of group A streptococci from excised tonsils

Griffith types	1	2	4	6	9	11	12	22	23	25	27	Unidentified
Number of strains	2	1	3	6	1	2	5	1	1	6	1	4

of recent infection and the level of circulating antibodies could be established.

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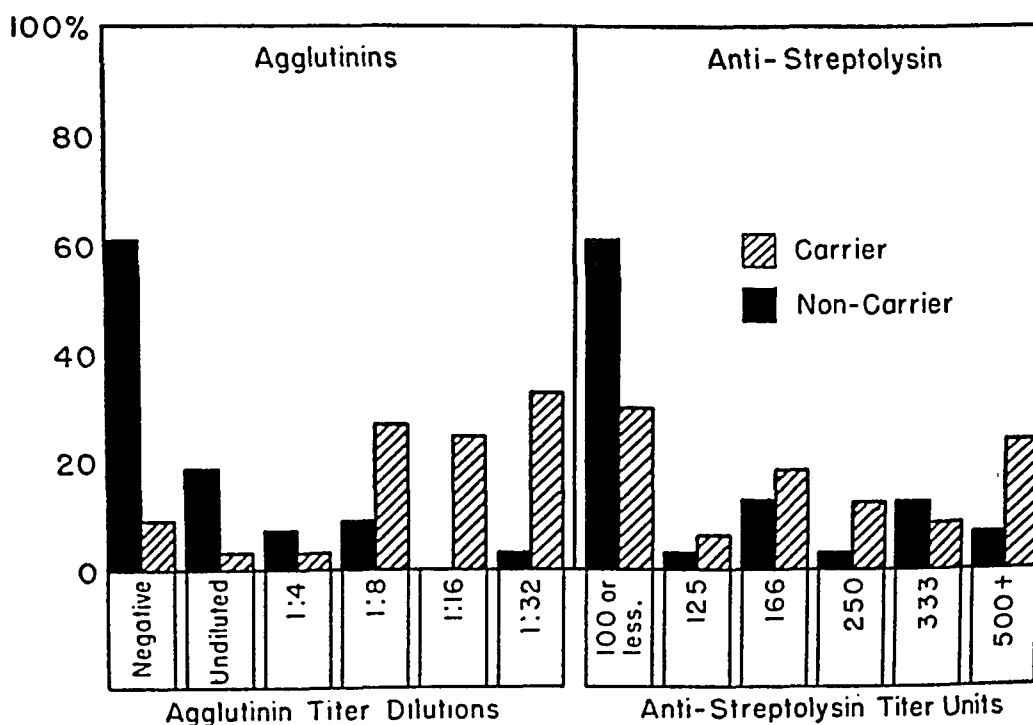


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maintainance of elevated levels of antistreptolysin and possibly of agglutinins.

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The interpretations of these results is difficult since two obvious causes for these variations in antibody level may be advanced. First, the presence of hemolytic streptococci in the tonsil may stimulate the maintenance of increased amounts of antibody or, second, carriers may simply have more recently undergone an active hemolytic streptococcus infection. The last hypothesis is not borne out by the histories obtained from the studied individuals or their families, but the value of such data in regard to respiratory infections in children is questionable.

A study of the antibody levels for a long time before and after tonsillectomy is necessary for the adequate evaluation of the importance of the carrier state in their maintenance. The follow-up observations in this study after operation are of interest, however, because of the consistent fall in antistreptolysin titer over a two-month period. A careful consideration of previous studies indicates that no such constant relationship exists if the antistreptolysin titers are followed over long periods, after an acute infection. While there is always a general tendency for the titers to return to normal with the passage of time, yet antistreptolysin titers greater than 400 units were present 6 months after an acute streptococcal respiratory infection in 49 per cent of the group studied by Mote and Jones (3). These same authors followed 12 cases at frequent intervals after an acute infection. Usually the antistreptolysin titer was well maintained for a long time or fell very slowly. In about half, the titers decreased rapidly after remaining at a high level for several months.

In the present group, the antistreptolysin titers fell markedly in 90 per cent of the cases during an interval of about 2 months. It seems reasonable to suppose that the stimulus to this constant occurrence, so unlike that observed by others, was the termination of the carrier state by tonsillectomy, and that the presence of Group A streptococci in the tonsil did contribute to the maintenance of increased amounts of antistreptolysin.

The high antistreptolysin titers discovered in a small number of non-carriers may be explained in three ways. Half of this group harbored strains of Groups C and G in the tonsil, and it has been shown elsewhere (6) that certain members of these groups are toxigenic and produce strep-

tolysin, antigenically similar to that of Group A. The presence of these organisms may have stimulated and maintained high levels of antistreptolysin. It is possible that in the others, a previous Group A carrier state was terminated spontaneously just before the individuals came under study, or that the cultural techniques failed in an occasional instance to recover the hemolytic streptococci resident in the tonsil.

A consistent fall of agglutinin titer after tonsillectomy was not observed and much more detailed study will be necessary to determine the importance of the carrier state for the maintenance of increased amounts of these antibodies, but it is possible that titers above 1:8 are stimulated by hemolytic streptococci in the nasopharynx, since these levels are rarely found in the sera of non-carriers.

The presence of large amounts of circulating antibacterial and antitoxic antibodies in the sera of Group A hemolytic streptococcus carriers cannot be interpreted as evidence that such individuals are immune to infection by these organisms, since the protective value of these substances is in no sense established. It is of great interest, however, that these evidences of potential immunity should be so constantly present in a group of individuals that has been previously demonstrated, by Bloomfield and Felty, to be extremely resistant to infection. Further studies for the purpose of clarifying these important points are in progress and will be described later.

#### SUMMARY

1. The Group A hemolytic streptococcus agglutinin and the antistreptolysin titers of the sera of 64 children were measured just previous to tonsillectomy.

2. Thirty-three of these individuals were demonstrated to be Group A carriers by culture of the excised tonsils.

3. The sera of carriers contained larger amounts of agglutinins and antistreptolysin than did that of non-carriers.

4. When the carrier state was terminated by tonsillectomy, there was a constant decline in antistreptolysin titer within 60 days, and a less regular fall in agglutinin titer.

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The presence of large amounts of circulating antibacterial and antitoxic antibodies in the sera of Group A hemolytic streptococcus carriers should not be interpreted as evidence that such individuals are immune to infection by these organisms since the protective value of these substances has no sense established. It is of great interest, however, that these evidences of potential immunity should be so constantly present in a group of individuals that has been previously demonstrated by Bloomfield and Felty, to be extremely resistant to infection. Further studies for the purpose of clarifying these important points are in progress and will be described later.

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## GROUP A HEMOLYTIC STREPTOCOCCUS ANTIBODIES II

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# THE ABSORPTION, EXCRETION, AND DISTRIBUTION OF PENICILLIN<sup>1</sup>

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In 1929, Fleming (1) described an active antibacterial substance, penicillin, obtained from the mold, *Penicillium notatum*. This substance has been shown to exhibit a marked antibacterial effect both *in vitro* (1, 2) and in the experimental animal (2). In man, the observations on the efficacy of penicillin as a therapeutic agent have been limited to those of Florey and his co-workers (3).

As a part of our investigation of the therapeutic effectiveness of penicillin in various infections, this study was undertaken to determine its absorption, excretion, and distribution when administered by various routes.

## MATERIALS AND METHODS

The subjects included normal volunteers and ward patients. The latter group were for the most part suffering from localized infections. Unless otherwise indicated, the urine and the blood non-protein nitrogen were normal in each subject. The majority of the studies were made in the fasting state; however, after the first 4 hours of observation fluids were not limited.

Penicillin<sup>2</sup> in the form of the sodium salt was dissolved in either distilled water or 0.85 per cent sodium chloride solution and passed through a Seitz filter to effect sterilization. The final concentration of all solutions of penicillin was 1,000 Florey units per cubic centimeter except that administered subcutaneously, which contained 200 Florey units per cubic centimeter of 0.85 per cent sodium chloride. The various solutions of penicillin were stored at 5° C. until time of use.

Penicillin was administered by the oral, intraduodenal, rectal, intravenous, subcutaneous, intramuscular, intrapleural, intra-articular, and intrabursal routes. The oral and rectal doses were administered in 200 cc. of tap water. Prior to rectal administration the subject was given a soap-and-water enema. Intraduodenal administration was effected through a Miller-Abbott tube, the position being checked by fluoroscopic examination. Intra-articular and intrabursal injections were made after aspira-

tion of a transudate. The gluteal muscles were used for intramuscular injections, and the medial aspect of the thigh for subcutaneous injections. Intrapleural injections were made in subjects with empyema, the exudate having been aspirated just prior to the administration of penicillin.

No serious toxic manifestations were caused by the administration of penicillin during the course of this study. The subjects complained of no untoward symptoms after intravenous injections of 5,000 to 40,000 Florey units. In one subject who received a constant intravenous drip of 102,500 Florey units in a period of 38 hours, no toxic reaction occurred. The intramuscular injection of 10,000 Florey units of penicillin in distilled water was attended by definite residual soreness, but such reactions were not encountered when the penicillin was dissolved in 0.85 per cent sodium chloride. The subcutaneous injection of 10,000 Florey units in 50 cc. of 0.85 per cent sodium chloride resulted in soreness and erythema, and the latter did not disappear for 12 to 24 hours. When a somewhat more dilute solution of penicillin was used, no erythema was noted. Penicillin has a very bitter taste, so that oral administration was somewhat unpleasant.

Samples of blood were withdrawn from each subject before and at frequent intervals after the dose of penicillin had been administered. The blood was then either defibrinated<sup>3</sup> or allowed to clot in sterile tubes. In both instances, the serum was separated by centrifugalization.

Urine was collected in a sterile container from all male subjects. In females, it was usually voided without sterile precautions except in a few subjects from whom the urine was obtained every 15 minutes by means of an indwelling catheter. In general, for a period of 24 hours after the penicillin had been given, all urine was collected as individual specimens.

Spinal fluid was obtained at varying intervals up to 195 minutes after the injection of penicillin. Joint fluid and exudate from the pleural cavity were obtained by aspiration.

All samples of body fluids were stored at 5° C., without the addition of any preservative, until the time of testing. If the sample was known to be contaminated, it was passed through a Seitz filter. In most cases, the determinations of penicillin were made on the same day the subject received the material. In a few subjects, the sam-

<sup>1</sup> Supported by a grant from the Johnson Research Foundation, New Brunswick, New Jersey.

<sup>2</sup> The penicillin used in this study was supplied through the courtesy of Dr. George A. Harrop, Squibb Institute for Medical Research, New Brunswick, New Jersey.

<sup>3</sup> The blood was defibrinated in many cases because a simultaneous study was made on the bactericidal power of whole blood following penicillin administration. This study will be reported at a later date.

ples were tested several days after the specimens were obtained.<sup>4</sup>

The method used to determine the concentration of penicillin in the samples of the various body fluids has been described previously (4). In brief, serial dilutions of 0.2 cc. of the unknown sample were made with 0.2 cc. plain broth through a series of 3 to 14 tubes. In addition, 0.5 cc. of the unknown sample was added to one tube. Similar dilutions of a known standard of penicillin, containing 20 Florey units per cc., were made. To all the tubes was added, then, 0.5 cc. of plain broth containing 1 per cent erythrocytes and from 1,000 to 10,000 hemolytic streptococci of a standard strain. The tubes were placed in an incubator at 37° C. for 18 to 24 hours and examined for visible growth. In general, those cultures showing no hemolysis were sterile. However, this was checked by streaking several dilutions on either side of the end point on blood-agar plates.

The concentration of penicillin in the unknown sample may then be determined by comparison with the standard control of penicillin.<sup>5</sup> It has been found that 0.0039 Florey unit is required to sterilize the culture of hemolytic streptococci contained in a total volume of 0.7 cc.

#### EFFECT OF INTRAVENOUS ADMINISTRATION ON THE SERUM CONCENTRATION AND URINARY EXCRETION

Figure 1 shows the concentrations of penicillin in the serum, and the cumulative excretion in the urine in a subject with an inlying catheter, following the injection of 20,000 Florey units. The

<sup>4</sup> If the specimen to be tested is kept at icebox temperatures, there is no appreciable loss of penicillin (4).

<sup>5</sup> The figures recorded in this paper on the concentration of penicillin in the various body fluids are subject to the error of dilution methods in general.

level in the serum rose rapidly, reaching a maximum immediately after the injection. Following this, there was a very rapid fall and at the end of 140 minutes, no penicillin could be detected. The rapid excretion into the urine is especially well demonstrated here, where 43 per cent of the injected dose was recovered within 1 hour after the injection. The concentration of penicillin was so high during this period that the urine was colored a bright yellow. In a few patients from whom frequent collections of urine were made, it was also noted that the period of greatest excretion of penicillin was accompanied by an increased volume of urine.

In all experiments in normal subjects, from 37 to 99 per cent of the intravenous dose was found in the urine, and the greatest amount was excreted in the first hour.

The effect on the concentration in the serum of varying the size of the dose of penicillin is illustrated in Figure 2. The single doses ranged from 5,000 to 40,000 Florey units. The greatest rise occurred immediately following the largest dose. Traces of penicillin were detected in the serum for only 30 to 40 minutes after 5,000 Florey units had been injected, whereas with amounts as large as 20,000 to 40,000 units, traces were observed for as long as 185 minutes after injection.

It is of some interest to point out that the spinal fluid of 3 patients (Subjects 4, 5, and 8, Table I) contained no penicillin after the injection of from 10,000 to 20,000 Florey units.

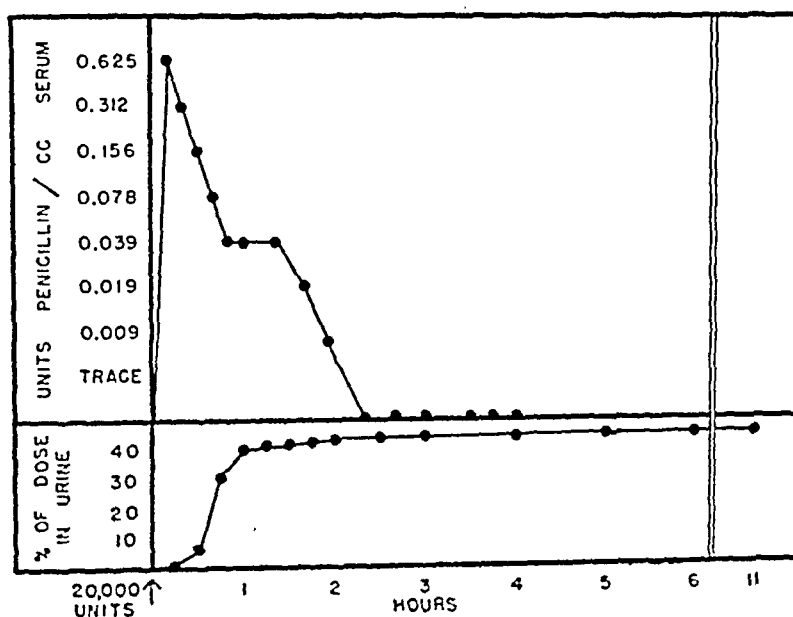


FIG. 1. RATE OF EXCRETION OF PENICILLIN FOLLOWING  
INTRAVENOUS ADMINISTRATION

TABLE I

*Serum concentrations and urinary excretion of penicillin after administration by various routes*

Route	Dose	Subject			Serum		Urine				Diagnosis and remarks
		Number	Age	Weight	Time *	Units	Time	Volume	Units	Units excreted (cumulative)	
Intra-venous	5,000	1	years 36	lbs. 139	minutes 5 20 30 40 70	per cc. 0.156 0.039 0.019 tr. 0	minutes	cc.	per cc.		Chronic osteomyelitis
Intra-venous	5,000	2	31	192	1 3 9 30 40	0.625 0.312 0.156 0.078 0	60 200 540	164 118 440	20.0 1.25 0.039	3,280 3,427 3,444	Normal subject
Intra-venous	5,000	11	28	152	1 3 7 12 20 30 40	1.25 0.312 0.156 0.156 0.039 tr. 0	60 655	97 379	40.0 0.625	3,880 4,117	Normal subject
Intra-venous	10,000	2	31	192	5 25 50 70 95 110	0.625 0.078 0.039 0.019 0.007 0	60 120 180 240	144 590 59 32	40.0 0.625 1.25 1.25	5,760 6,127 6,200 6,232	Normal subject
Intra-venous	10,000	3	40	118	2 10 20 30 37 45 60 75 90 105	1.25 0.312 0.156 0.156 0.078 0.039 0.019 0.019 0.007 0	5 240†	3 245	0 16	0 3,955	Normal subject
Intra-venous	10,000	4	55	111	5 30 90 150 210	0.625 0.078 0.019 0.019 0.019					Spinal fluid at 5, 15, 30, and 45 minutes contained no penicillin
Intra-venous	10,000	5	23	101	5 105	5.0 0					Patient 5 months pregnant. Spinal fluid at 95 minutes contained no penicillin
Intra-venous	20,000	6	52	153	1 10 22 45 125 150 185	2.5 0.625 0.156 0.156 0.019 0.007 tr.	60 120 180 240 300 345	18.4 14.5 79.6 28.9 25.4 21.6	175.4 129.3 29.1 6.2 1.6 1.9	3,228 5,103 7,424 7,603 7,644 7,688	Brain tumor
Intra-venous	20,000	7	24	112	5 90 180	10.0 0.156 0	240	124.0	160.0	19,840	Subacute bacterial endocarditis

\* All times recorded are from the end of penicillin administration.

† No further urine specimens obtained.

Serum and urine concentrations are recorded only to the time when penicillin was no longer detected. Tr. = trace of penicillin.

TABLE I—*Continued*

Route	Dose	Subject			Serum		Urine				Diagnosis and remarks
		Number	Age	Weight	Time *	Units	Time	Volume	Units	Units excreted (cumulative)	
Intra-venous	20,000	8	years 48	lbs. 133	minutes	per cc.	minutes	cc.	per cc.		Spinal fluid at 135, 165, and 195 minutes contained no penicillin
					10	1.25					
					25	0.312					
					50	0.078					
					75	0.039					
					105	0.019					
					150	tr.					
Intra-venous	20,000	9	35	124	180	0					Boeck's sarcoid
					10	0.625	15	4.2	0	0	
					20	0.312	30	9.4	125.0	1,174	
					30	0.156	45	21.0	250.0	6,424	
					40	0.078	60	55.4	31.2	8,152	
					50	0.039	75	93.0	2.68	8,478	
					60	0.039	105	113.9	1.56	8,655	
					82	0.039	120	34.9	1.83	8,734	
					100	0.019	150	34.8	3.12	8,847	
					115	0.007	165	14.2	3.13	8,891	
					140	0	210	40.1	1.56	8,954	
							240	37.2	0.78	8,984	
							300	132.7	0.20	9,011	
							360	96.9	0.08	9,019	
							660	217.4	0.04	9,028	
Intra-venous	40,000	10	33	155	1	2.5	125	181.0	80.0	14,480	Chronic osteomyelitis
					3	1.25	225	170.0	1.25	14,692	
					9	0.625	330	36.0	0.312	14,702	
					13	0.312	400	36.0	0.625	14,724	
					25	0.156	480	52.0	0.078	14,728	
					42	0.078					
					65	0.078					
					80	0.039					
					95	0.019					
					120	0.007					
					140	0.007					
					180	0					
Subcutaneous	10,000	12	21	163	5	0	120	285.0	1.25	356	Rheumatic fever
					20	0	300	135.0	10.0	1,706	
					35	0	540	205.0	1.25	1,962	
					60	tr.	660	123.0	0.078	1,972	
					85	tr.					
					120	0.007					
					150	0.007					
					190	0.007					
					255	0.007					
					300	tr.					
Subcutaneous	10,000	13	30	127	5	0	70		5.0	900	Normal subject
					15	0	250		80.0	6,500	
						0	380		7.0	8,200	
						0	540		2.5	8,427	
						0	720		0.312	8,462	
						0	1,470		0	8,462	
						tr.					
						tr.					
Intramuscular	10,000			139							osteomyelitis

Route	Dose	Subject			Serum		Urine				Diagnosis and remarks
		Number	Age	Weight	Time *	Units	Time	Volume	Units	Units excreted (cumulative)	
Intra-muscular	10,000	14	years 28	lbs. 154	minutes 5 12 25 45 70 100 120 135 160 185	per cc. 0.078 0.078 0.078 0.078 0.015 tr. tr. tr. tr. 0	minutes 100 250 390 1,320	cc. 415.0 238.0 598.0 380.0	per cc. 20.0 5.0 0.156 0	8,300 9,490 9,583 9,583	Rheumatoid arthritis
Intra-articular (knee joint)	10,000	12	21	163	5 15 25 45 55 70 95 130 180 210 240	0 0 0.007 0.007 0.007 0.019 0.019 0.019 0.019 0.007 0.007	120 240 420 540 780	208.0 237.0 190.0 176.0 465.0	2.5 5.0 2.5 0.312 0.019	520 1,705 2,108 2,163 2,171	Rheumatic fever
Intra-pleural	10,000	19	40		15 30 60 90 120 150 210 255 300 375 435	tr. 0.007 tr. tr. 0.007 0.007 tr. tr. 0.007 0.007 0.007	275 600 1,020	525.0 575.0 336.0	2.5 0.625 0.625	1,312 1,671 1,881	Empyema fluid at 22 hours contained 0.78 unit per cc.
Intra-pleural	5,000	21	13	78	60 120 175 245	0 0 0 0	255 420 660 1,500	407.0 228.0 131.0 173.0	5.0 0.625 0.312 0.039	2,035 2,177 2,217 2,224	Chronic empyema
Intra-pleural	30,000	20	16	123	30 90 135 225 380 460	0 0.007 0.019 0.039 0 0.007	120 305 465 705 900 1,140	129.0 135.0 126.0 183.0 135.0 175.0	1.25 10.0 5.0 5.0 1.25 1.25	161 1,511 2,141 3,056 3,225 3,444	Injected into empyema cavity. At 24 hours empyema fluid contained 3.12 units per cc.
Sinus tract	12,000	22	21	132	45 80 110 150	0.039 0.019 tr. 0	485 720 1,200	346.0 112.0 173.0	0.312 0.156 0.039	108 125 132	Chronic osteomyelitis
Intra-bursal (suprapatellar)	10,000	14	28	154	15 30 50 70 85 115 145 190	0 0 0 0 0 0.007 tr. 0	120 240 300 360 480 810	363.0 91.0 54.0 324.0 548.0 325.0	5.0 20.0 10.0 0.312 0.156 0.078	1,815 3,635 4,175 4,276 4,361 4,386	Rheumatoid arthritis

TABLE I—Continued

Route	Dose	Subject			Serum		Urine				Diagnosis and remarks
		Number	Age	Weight	Time *	Units	Time	Volume	Units	Units excreted (cumulative)	
Intra-venous	20,000	8	years 48	lbs. 133	minutes	per cc.	minutes	cc.	per cc.		Spinal fluid at 135, 165, and 195 minutes contained no penicillin
					10	1.25					
					25	0.312					
					50	0.078					
					75	0.039					
					105	0.019					
					150	tr.					
					180	0					
Intra-venous	20,000	9	35	124	10	0.625	15	4.2	0	0	Boeck's sarcoid
					20	0.312	30	9.4	125.0	1,174	
					30	0.156	45	21.0	250.0	6,424	
					40	0.078	60	55.4	31.2	8,152	
					50	0.039	75	93.0	2.68	8,478	
					60	0.039	105	113.9	1.56	8,655	
					82	0.039	120	34.9	1.83	8,734	
					100	0.019	150	34.8	3.12	8,847	
					115	0.007	165	14.2	3.13	8,891	
					140	0	210	40.1	1.56	8,954	
							240	37.2	0.78	8,984	
							300	132.7	0.20	9,011	
							360	96.9	0.08	9,019	
							660	217.4	0.04	9,028	
Intra-venous	40,000	10	33	155	1	2.5	125	181.0	80.0	14,480	Chronic osteomyelitis
					3	1.25	225	170.0	1.25	14,692	
					9	0.625	330	36.0	0.312	14,702	
					13	0.312	400	36.0	0.625	14,724	
					25	0.156	480	52.0	0.078	14,728	
					42	0.078					
					65	0.078					
					80	0.039					
					95	0.019					
					120	0.007					
					140	0.007					
					180	0					
Subcutaneous	10,000	12	21	163	5	0	120	285.0	1.25	356	Rheumatic fever
					20	0	300	135.0	10.0	1,706	
					35	0	540	205.0	1.25	1,962	
					60	tr.	660	123.0	0.078	1,972	
					85	tr.					
					120	0.007					
					150	0.007					
					190	0.007					
					255	0.007					
					300	tr.					
Subcutaneous	10,000	13	30	127	5	0	70	182.0	5.0	900	Normal subject
					15	0	250	70.0	80.0	6,500	
					40	0	380	85.0	20.0	8,200	
					60	0	540	91.0	2.5	8,427	
					80	0	720	112.0	0.312	8,462	
					100	0	1,470	226.0	0	8,462	
					115	tr.					
					135	tr.					
					155	tr.					
					180	tr.					
					205	0					
Intramuscular	10,000	1	36	139	5	0.039					Chronic osteomyelitis
					30	0.078					
					60	0.078					
					90	0.039					
					120	0.007					
					150	0					

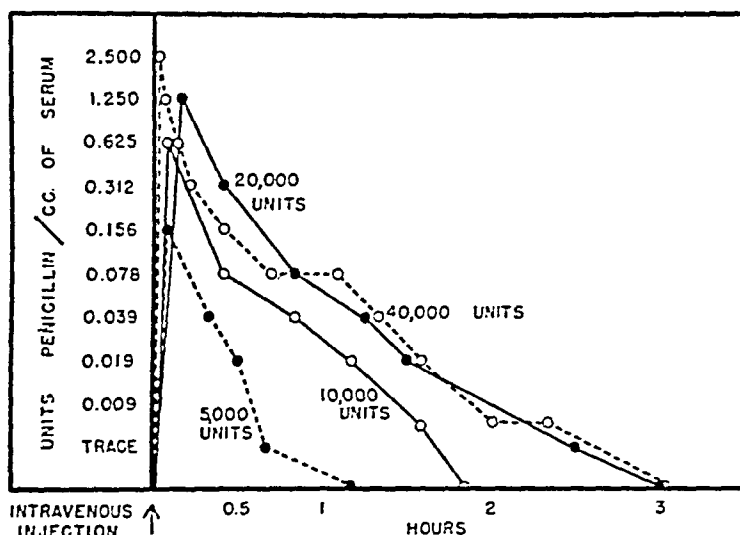


FIG. 2. SERUM CONCENTRATIONS FOLLOWING VARYING DOSES OF PENICILLIN

#### EFFECT OF VARIOUS PARENTERAL ROUTES OF ADMINISTRATION ON SERUM CONCENTRATION AND URINARY EXCRETION

In contrast to the immediate rise and very rapid fall in the level of penicillin in the serum following an intravenous injection, were the concentrations obtained after intramuscular and subcutaneous injections (Figure 3). After an intramuscular injection of 10,000 Florey units, there was a rather rapid rise in the serum concentration, which, however, did not reach as high a level as that obtained after an intravenous dose. The concentration in the serum then tended to remain at the peak height for 30 to 45 minutes and

thereafter decreased gradually. In 2 subjects (Numbers 1 and 14), the last trace was detected at 120 and 160 minutes, respectively. Urinary excretion was not prolonged in Subject 4.

The subcutaneous injection of 10,000 Florey units resulted in a rather prolonged delay in the appearance of penicillin in the blood stream. In 2 subjects (Numbers 12 and 13), it was first detected in the serum at 85 and 115 minutes, respectively. Its concentration in the plasma never reached the high levels obtained by either intravenous or intramuscular injection. Excretion in the urine was definitely delayed.

#### ABSORPTION AND EXCRETION FROM BODY CAVITIES

In Subject 12, 10,000 Florey units of penicillin were injected into the right knee joint. Penicillin was first detected in the serum 25 minutes later and reached a maximum height of 0.019 Florey unit per cc. at 70 minutes. The excretion in the urine was similar to that observed after subcutaneous injections in that it was delayed. A total of 21 per cent of the administered dose was found in the urine. At the end of 780 minutes, the knee was again aspirated and the fluid thus obtained was found to contain 0.039 Florey unit per cc.

In another subject (Number 14), 10,000 Florey units were injected into the suprapatellar bursa after it had been aspirated. Penicillin was first detected in the serum at 115 minutes. There was

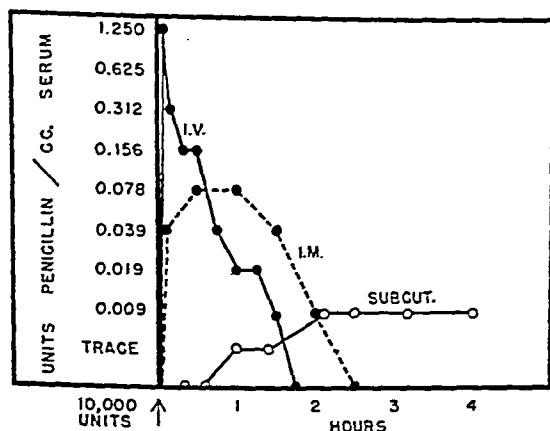


FIG. 3. CONCENTRATION OF PENICILLIN IN BLOOD SERUM FOLLOWING PARENTERAL INJECTION



TABLE I—Continued

Route	Dose	Subject			Serum		Urine				Diagnosis and remarks
		Number	Age	Weight	Time *	Units	Time	Volume	Units	Units excreted (cumulative)	
Oral	10,000	15	years 17	lbs. 149	minutes	per cc.	minutes	cc.	per cc.		Idiopathic epilepsy
					10	0	90	163.0	2.5	407	
					25	tr.	180	114.0	5.0	977	
					40	0.007	270	532.0	0.078	1,018	
					60	0.007	420	582.0	0	1,018	
					80	0.007	480	280.0	0	1,018	
					100	0.007					
					130	tr.					
Oral	20,000	16	21	142	5	0	60	300.0	0.312	93	Inguinal hernia
					30	0	300	432.0	1.25	633	
					70	0	600	255.0	0.039	643	
					95	0	840	163.0	0	643	
					120	0					
					165	0					
					210	0					
Oral	20,000	16	21	142	5	0	165	200.0	2.5	500	Given 4 grams of sodium bicarbonate 10 minutes before administration of penicillin
					18	0	325	243.0	1.25	803	
					30	0.007	365	90.0	2.5	1,028	
					45	0.039	560	134.0	0.078	1,038	
					65	0.007	705	127.0	0	1,038	
					85	0					
Duo-denal	10,000	17	42	199	5	0.007	240	557.0	2.5	1,392	Menorrhagia
					15	0.039	330	171.0	1.25	1,606	
					30	0.019	465	330.0	0.019	1,612	
					45	0.007					
					60	tr.					
					75	0					
Duo-denal	20,000	18	35	128	5	0	55	555.0	2.5	1,387	Psychoneurosis
					15	0.039	215	156.0	2.5	1,777	
					25	0.039	285	73.0	2.5	1,958	
					35	0.039	495	95.0	0.625	2,017	
					45	0.019					
					55	0.019					
					80	tr.					
Rectal	10,000	1	36	139	10	0	120	111.0	2.5	277	Chronic osteomyelitis
					45	0.007	240	80.0	5.0	677	
					90	0.007	360	108.0	2.5	947	
					120	0.007	480	80.0	2.5	1,147	
					150	tr.	540	135.0	0.625	1,231	
					180	tr.					
					225	tr.					
					270	0					
Rectal	20,000	1	36	139	5	0	50	454.0	0.078	35	Chronic osteomyelitis
					20	0.007	120	302.0	0.625	223	
					40	0.007	240	88.0	5.0	663	
					60	tr.	360	135.0	1.25	831	
					80	0	480	88.0	1.25	941	
					100	0.007	600	94.0	1.25	1,058	
					120	0.007					
					150	0.007					
					180	0.007					
					240	tr.					
					270	0.007					
					300	tr.					
					360	0					

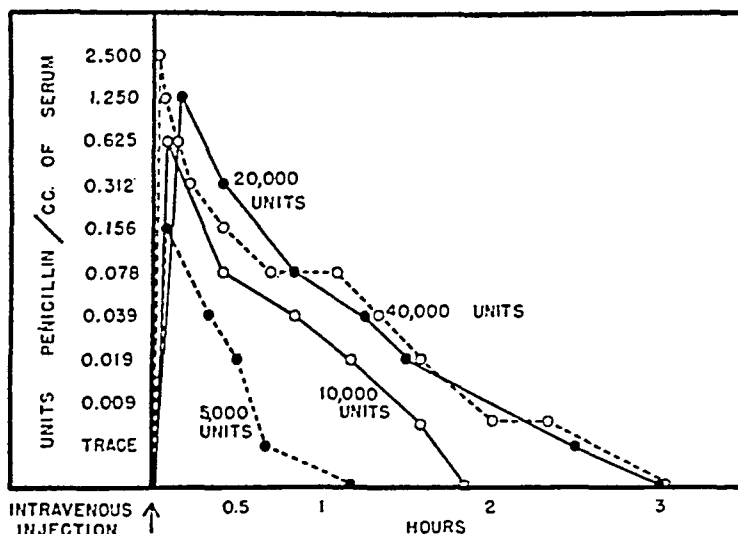


FIG. 2. SERUM CONCENTRATIONS FOLLOWING VARYING DOSES OF PENICILLIN

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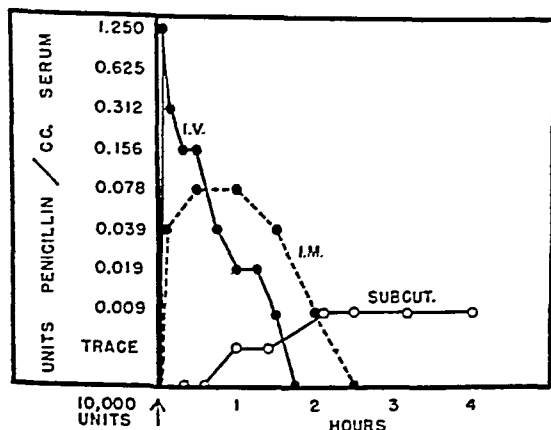


FIG. 3. CONCENTRATION OF PENICILLIN IN BLOOD SERUM FOLLOWING PARENTERAL INJECTION

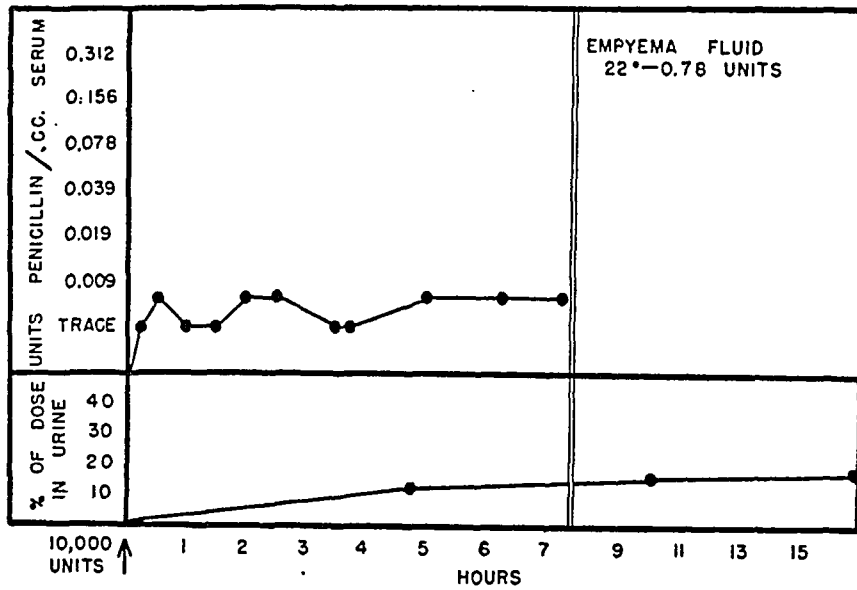


FIG. 4. ABSORPTION AND EXCRETION OF PENICILLIN FROM EMPYEMA CAVITY

a delay in its excretion in the urine, but the total amount excreted was 43 per cent.

Figure 4 shows the results obtained in Subject 19 following the injection of 10,000 Florey units directly into an empyema cavity. The cavity was aspirated and the penicillin then injected through the same needle. Within 15 minutes, a trace of penicillin was present in the serum, and for a total of 420 minutes, all samples of plasma contained a trace to 0.007 Florey unit per cc. The excretion in the urine was somewhat delayed. At the time of surgical drainage, 22 hours after the injection, a sample of the exudate contained 0.78 Florey unit per cc.

Subject 21 received only 5,000 Florey units which were injected through a draining sinus into an old empyema cavity. In this subject, no penicillin was detected in the serum; however, 44 per cent of the dose injected was found to be excreted in the urine. On another occasion, the same subject excreted 42 per cent of 5,000 Florey units which had been injected into the sinus. Following this, the patient received 3 injections of 10,000 Florey units and the subsequent excretion was found to be 33, 4, and 13 per cent, respectively.

One subject (Number 20) who developed an empyema following a lobectomy received 30,000 Florey units in a single dose. There was a rather long delay in the appearance of the substance in the blood stream and likewise a delay in its excretion in the urine. The total excretion amounted

to 11 per cent of the administered dose. Aspiration of the cavity 24 hours after injection showed that the exudate still contained 3.12 units per cc.

Similar results were obtained when penicillin was injected into a chronic sinus extending from the left buttock up to the lower pole of the left kidney (Subject 22). Penicillin appeared in the serum in this patient although the urinary excretion amounted to only 1 per cent. This may be partially explained by the fact that after the first 4 hours of this study, the patient moved about in bed, which allowed the penicillin to drain out through the sinus.

#### ABSORPTION AND EXCRETION AFTER ENTERAL ADMINISTRATION

The absorption and excretion of penicillin were studied after oral, intraduodenal, and rectal administration of 10,000 to 20,000 Florey units. Figure 5 demonstrates the concentrations obtained in the serum of the 3 subjects (Numbers 17, 15, and 1) following the administration of 10,000 Florey units by the intraduodenal, oral, and rectal routes, respectively. After the intraduodenal administration, there was a greater and more rapid rise in the concentration in the serum than was observed following rectal or oral administration, the curve being similar to that obtained after intravenous or intramuscular injection in that there was a rather abrupt rise, a short duration of the peak concentration, and a rapid

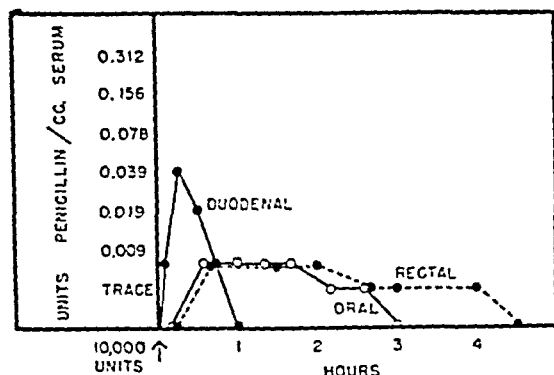


FIG. 5. CONCENTRATION OF PENICILLIN IN BLOOD SERUM AFTER ENTERAL ADMINISTRATION

disappearance of penicillin from the blood stream. Although the excretion was apparently more rapid following intraduodenal administration, the total amount found in the urine was of the same order as that found after oral and rectal administration, the amount excreted being 10, 16, and 12 per cent following the oral, intraduodenal, and rectal doses, respectively.

In Subject 16, 20,000 units of penicillin were given by mouth after 12 hours of fasting, and again after a 12-hour fast and the ingestion of 4 grams of sodium bicarbonate, 10 minutes prior to the dose of penicillin. In the first instance, no penicillin appeared in the serum; following sodium bicarbonate, small amounts were detected for a period of 35 minutes. The excretion in the two

tests was of the same order, being 3 and 5 per cent of the administered dose.

#### EFFECT OF RENAL FUNCTION ON EXCRETION

In this study, 3 patients were given a standard dose of 10,000 units of penicillin intravenously. Brief case histories are presented below.

##### Subject A

This patient, a female, 34 years old, had been observed over a period of 6 years with chronic progressive renal failure. At the time penicillin was administered, the blood pressure was 180/106. Urine examinations showed a specific gravity of 1.003 to 1.013, albumin was present in all specimens, and many leukocytes and erythrocytes were seen on microscopic examination. The red cell count was 2,770,000 and the hemoglobin content was 56 per cent. The non-protein nitrogen was 134 mgm. per 100 cc., and the urea clearance 10 per cent.

##### Subject B

This female, 25 years of age, who had entered the hospital because of an attack of acute disseminated lupus erythematosus at the age of 23, again entered the hospital. The blood pressure was 120/78 and the heart was not enlarged. Anemia was moderate, with 3,810,000 red cells and a hemoglobin concentration of 70 per cent. The non-protein nitrogen was 168 mgm. per 100 cc., and the urea clearance 9 per cent. The specific gravity of the urine ranged from 1.007 to 1.016 and albumin was present in all specimens.

##### Subject C

A normal subject, 31 years old. (Subject 2, Table I.)

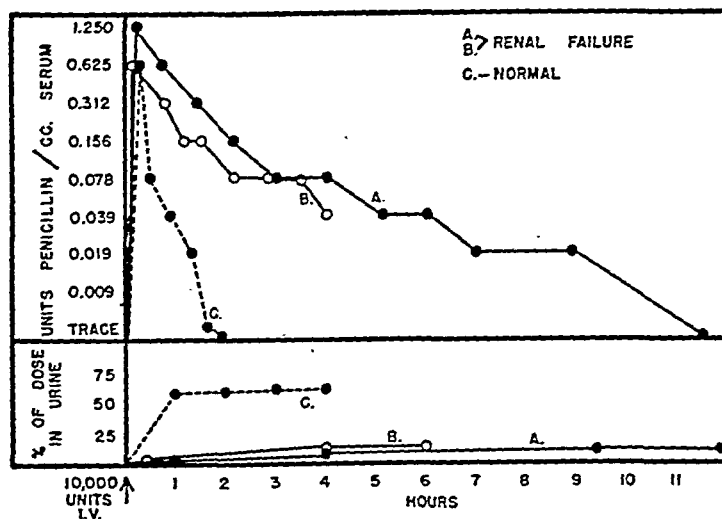


FIG. 6. EFFECT OF NORMAL AND DEPRESSED RENAL FUNCTION ON EXCRETION OF PENICILLIN

TABLE II  
*Distribution of penicillin between blood plasma and red blood cells*

Unknown sample *	Culture †	Growth in serial dilutions of unknown samples											
		Undiluted sample 0.2 cc.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Whole blood	B.B. B.A.P.	0	0	0	0	0	0	0	0	± ++	+ ++	+	+
Serum	B.B. B.A.P.	0	0	0	0	0	0	0	0	0	± ++	++ ++	++
Cells	B.B. B.A.P.	0	0	0	0	0	++	++	++	++	++	++	++
Hemolyzed cells	B.B. B.A.P.		0	0	0	0	++	++	++	++	++	++	++

\* Unknown sample = Defibrinated blood which has been exposed for 19 hours to an unknown amount of penicillin at ice box temperature. Control studies not included in the table have established that 0.0039 Florey unit is the smallest amount of penicillin required to sterilize the culture. The number of units per 0.2 cc. of the unknown sample may then be determined by multiplying 0.0039 by the dilution factor. In the whole blood, there are 0.5 Florey unit per 0.2 cc. or 2.5 units per cc.

† Culture: B.B. = blood broth. B.A.P. = subculture on blood agar plates

0 = no visible growth or hemolysis.

+ and ++ = degree of growth.

Inoculum = 0.5 c.c. of blood broth containing 2,700 streptococci.

Figure 6 shows the results obtained in the 2 patients with renal failure, Subjects A and B, and also in a normal volunteer, Subject C. It is at once apparent that the concentration of penicillin in the serum remained elevated for a prolonged period of time in the 2 subjects with renal failure, whereas all traces of penicillin had disappeared from the blood in the normal subject within 110 minutes.

The prolonged antibacterial effect noted in the serum of the 2 patients is most likely explained by the diminished excretion of penicillin. In the normal subject, 57 per cent of the administered dose was excreted within the first hour, whereas Subject B excreted only 14 per cent in 6 hours, and Subject A, 12 per cent in 13 hours.

#### DISTRIBUTION OF PENICILLIN BETWEEN BLOOD PLASMA AND RED CELLS

In order to determine the distribution of penicillin between red cells and blood plasma, whole defibrinated blood containing varying amounts of penicillin was allowed to stand for 2 to 19 hours with occasional agitation. Following this preparation, determinations for penicillin were made on the whole blood, serum, intact cells, and hemolyzed cells.

Table II demonstrates the results obtained in one such experiment. Control observations, using a standard solution of penicillin and made simultaneously with the test, showed that 0.0039 Florey unit was the smallest amount of penicillin which would sterilize the culture. Furthermore, if 0.2 cc. of whole blood, serum, or cells was first added to the standard solution of penicillin and serial dilutions were then made, the end-point was not altered, indicating that these 3 substances do not inhibit the antibacterial action of penicillin.

When the defibrinated whole blood which had been exposed to penicillin for a period of 19 hours was tested, it was found to kill hemolytic streptococci through a dilution of 1:128. The serum sterilized the culture through a dilution of 1:256, and the cells, both intact and hemolyzed, through a dilution of 1:8. Translating this into the number of units per cc., one finds that the whole blood, serum, and cells contained 2.5, 5, and 0.156 Florey units per cc., respectively. The fact that the serum contained twice the concentration of the whole blood sample suggested that the cells contained little or no penicillin.

In Table III, the results of several experiments are recorded; it is apparent that the plasma trapped in the cell mass (5) does account for a significant part of the penicillin detected in the

TABLE III

*Distribution of penicillin between red blood cells and blood plasma*

Hours exposed to penicillin	Hematocrit	Units of penicillin per cc.				
		Whole blood	Plasma	Cells (A)	In cell mass (accounted for by trapped plasma) (B)	True concentration in cells (A-B)
2	45.2	2.5	5.0	0.312	0.187	0.125
2	45.2	2.5	5.0	0.312*	0.187	0.125
19	42.0	2.5	5.0	0.156	0.174	-0.018
19	42.0	10.0	10.0	0.625	0.438	0.187
14	45.0	5.0	10.0	1.250	0.373	0.877
14	45.0	1.25	2.5	0.312	0.093	0.219

\* Cells hemolyzed before determination of concentration of penicillin.

cells. The true concentration in the cells, as listed in the last column, shows that the amount of penicillin that penetrates the cells is exceedingly small and is usually less than 10 per cent of the plasma concentration.

#### DISCUSSION

Few studies on the absorption and excretion of penicillin have been made in man. Florey (3) found that no deleterious effects occurred after the intravenous injection of 200 mgm. (about 8,000 Florey units). This was the largest amount administered as a single dose. Following the injection, the initial high value of penicillin in the blood declined to a discernible trace 125 minutes later. Furthermore, the excretion of penicillin in the urine was invariably less than the administered dose. In 2 subjects, 50 and 68 per cent of the antibacterial activity was found in the urine.

In the studies reported here, penicillin was injected intravenously, in doses ranging from 5,000 to 40,000 Florey units. The results agree with the observations of Florey (3) in that penicillin was detected in the peripheral blood and that the active substance was excreted in the urine in amounts less than the dose injected. The concentration reached in the blood plasma was related to the size of the dose injected, the highest concentration being 10 Florey units per cc. of serum. Following the initial rise in the serum concentration, there was a rapid fall. The rapid clearing of the substance from the blood plasma is explained by its excretion in the urine.

Of interest are the results obtained when penicillin was administered by enteral routes. The absorption following oral administration was poor, as demonstrated both by low concentrations obtained in the blood plasma and by the small amounts excreted in the urine. In the 3 tests where penicillin was given orally, the average excretion was 8.6 per cent. The statement (3) that acid destroys penicillin may account for these results; however, in Subject 16, the administration of alkali just prior to ingestion of penicillin did not significantly alter its absorption or excretion. It seems unlikely from these limited observations that oral administration will give adequate concentrations in the blood plasma for the treatment of infections.

Absorption from the intestine was greatest following intraduodenal administration. Here the penicillin appeared in the blood plasma and reached its maximum level within 5 to 15 minutes after the injection. The curve of plasma concentrations was similar to that obtained after intramuscular injection. The excretion in the urine averaged 18 per cent of the administered dose.

Rectal absorption of penicillin was poor, the maximum concentration in the blood plasma being 0.007 Florey unit per cc. following the injection of either 10,000 or 20,000 Florey units. Excretion in the urine was also low, averaging 11 per cent of the injected dose. This decreased excretion may be due entirely to poor absorption; however, Florey (3) observed that feces inactivate penicillin, and more recently it has been demonstrated that extracts of *Escherichia coli* will inhibit the action of penicillin (6).

The fact that penicillin may be recovered in the urine suggested that the rapid fall in serum concentration following intravenous injection was due primarily to excretion by the normal kidney. This was well demonstrated to be the case in those subjects in whom frequent collections of urine were made. In such subjects, the largest amount of penicillin was excreted during the first hour after the intravenous injection. Indeed, the substance appeared to act as a diuretic in these experiments.

From the study of the 2 subjects with renal failure, further evidence was obtained in support of the view that the chief factor causing the rapid clearing of blood is the excretion in the urine.

In these patients, a relatively high concentration of penicillin was maintained in the blood plasma for as long as 9 hours after the intravenous injection of 10,000 Florey units.

Little is known concerning the distribution of penicillin in the body fluids. Florey (3) demonstrated in animals that after an intravenous injection, penicillin could be detected in whole blood, bile, and saliva, but not in tears or pancreatic juice. In the present studies, after a single intravenous injection of 10,000 to 20,000 Florey units, no penicillin was found in the spinal fluid. In one subject with osteomyelitis, who was being treated with a constant intravenous drip of 2,500 to 3,000 Florey units per hour, no penicillin was detected in the spinal fluid after 24 hours of such therapy. None was found in the tears or saliva of this patient. However, penicillin injected intrathecally (7) was absorbed and excreted in the urine both in normal subjects and in patients with meningitis. No observations have been made concerning the diffusion of penicillin from the blood stream into the spinal fluid in subjects with meningitis.

The studies on the distribution of penicillin between blood plasma and erythrocytes showed that minimal quantities penetrated the red cells.

The observations made by Florey (3), and confirmed by these studies, that the excretion of penicillin in the urine was always less than the amount administered suggests that it was destroyed or inactivated in the body. In general, after intravenous injection, excretion in the urine accounted for about 60 per cent of the administered dose. When penicillin was administered by routes that result in slow absorption, the percentage recovery in the urine was even lower and, further, in those patients with renal failure, the total excretion was extremely low. These latter two observations support the view that penicillin is inactivated in the body.

The cause of this apparent loss of penicillin is not explained. This study showed that red cells and plasma did not inhibit its action. Further, its incubation with slices of liver, kidney, spleen, brain, muscle, lymph gland, intestine, lung, and bile, caused no destruction of the penicillin (3). Although elevated temperatures will destroy penicillin activity, it is unlikely that body temperatures

cause a significant loss of penicillin during the short period it remains in the body.

The maintenance of an adequate concentration of penicillin in the body is necessary if therapy in man is to be successful. Factors of importance in deciding the size of the dose and the route of administration are the type of infecting organism and the site of infection. Micro-organisms vary in their susceptibility to the action of penicillin (3). In general, hemolytic streptococci and pneumococci are extremely sensitive. From *in vitro* tests, we have found that as little as 0.0039 Florey unit was required to kill from 1,000 to 100,000 hemolytic streptococci, whereas about 0.03 Florey unit was required to sterilize similar numbers of staphylococci (8). Studies on the bactericidal power of whole blood demonstrated that plasma concentrations of 0.03 and 0.3 Florey unit per cc. were required to cause maximal killing against the hemolytic streptococcus and *Staphylococcus aureus*, respectively (8). Somewhat lower concentrations in the plasma were associated with a definite bacteriostatic effect.

The location of the infection is of utmost importance in determining the route of administration, since it is evident from the studies reported here that penicillin is excreted rapidly and does not diffuse readily. Thus, if a localized infection is being treated by intravenous therapy, the blood supply to the area must be adequate if sterilization is to be effected. It is advisable, therefore, to give penicillin locally rather than intravenously in infections of the pleural and joint cavities. In generalized infections, such as bacteremia, intravenous or intramuscular therapy is indicated.

#### SUMMARY

Data are presented concerning the blood concentration and urinary excretion of penicillin after the administration of 5,000 to 40,000 Florey units by several routes.

Intravenous injection of penicillin resulted in high initial concentration in the blood plasma which was followed by an abrupt fall. Traces of penicillin were found in the blood for 30 to 210 minutes after the injection, the length of time depending on the amount administered. The sharp fall noted in the serum concentration immediately after injection was associated with an increased

excretion in the urine. The average excretion after intravenous injection was 58 per cent of the administered dose.

Penicillin was rapidly absorbed when given intramuscularly and slowly absorbed after subcutaneous injections. Excretion in the urine was rapid following intramuscular injections and delayed after subcutaneous injections.

Absorption from the body cavities was delayed, and this was reflected in the slow excretion of penicillin by the kidneys. The total amount found in the urine was somewhat lower than that obtained following intravenous injection. Fluid aspirated from the pleural and joint cavities, 22 and 13 hours after the injection, showed appreciable amounts of penicillin remaining.

Administration of penicillin by enteral routes showed that absorption from the duodenum was rapid, whereas oral and rectal doses were poorly absorbed. These findings may be explained by the inactivating effect on penicillin of acid and *Escherichia coli*. After oral, intraduodenal, and rectal administration, the average amount excreted in the urine was extremely small.

In the presence of renal failure, penicillin was not excreted rapidly, and as a result, high concentrations were maintained in the blood stream after intravenous injections.

Studies on the distribution of penicillin showed that the substance failed to penetrate the red cells

in significant amounts. In general, the average concentration found in erythrocytes was less than 10 per cent of the plasma concentration. No penicillin was found in the spinal fluid, saliva, or tears, in subjects receiving it intravenously.

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# THE CHANGES WHICH ALTER RENAL OSMOTIC WORK

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## INTRODUCTION

Although realization that the kidney must do osmotic work antedates the greater part of our present knowledge of renal function, the implications of this requirement have not received the consideration they deserve, either by physiologists or by clinicians. Physiologically, there is, for instance, in terms of osmotic work, an optimal urine volume for removal of a given quantity and assortment of substances in urine. Clinically, there is the consideration of the consequences of the inability on the part of disabled kidneys to do sufficient osmotic work. Since, by adjustments of food and water intake, the kidneys' task can be changed in directions which will reduce osmotic work, regard for osmotic capacity is obviously of therapeutic importance. Reduction of the amount of a substance ingested does not necessarily reduce the osmotic work done by the kidney.

von Rhorer (1), in 1905, obtained an equation for the work required to excrete a single urinary component and this equation has been used by Addis (2) in studying experimental nephritis. In the present paper, it is undertaken to derive and examine the equation which will evaluate the osmotic work required for the simultaneous removal or non-removal of several substances and which will permit estimation of the adjustments that will produce reduced osmotic work.

## DERIVATION OF THE BASIC EQUATIONS

The osmotic work of the kidney is done in converting the glomerular filtrate into urine. Inasmuch as this is an isothermal process, the reversible or minimal work required will be only a function of the initial and final states, and thus the work calculated over any convenient reversible path will equal the work for any other reversible path. This is illustrated by the fact that the equation giving the amount of work may be derived by considering several different hypothetical processes resulting in the formation of urine.

The equation may be derived by an extension of von Rhorer's method. A theoretical justification of the equation, as used by Barcroft, has been given by R. G. Dickinson and R. C. Tolman in an article by Borsook and Winegarden (3). The following derivation employs only one of several convenient paths which suggest themselves. We hypothesize the following simple process. One liter of glomerular filtrate is considered to form one liter of urine by the addition of certain substances to it, and the removal of other substances by the tubules.<sup>1</sup>

The amount of work  $dE$  required to produce an infinitesimal change in volume  $dv$  against a pressure  $p$  is given by

$$dE = -p dv.$$

By van't Hoff's law we have  $p = cRT$ , where  $c$  is the osmolar concentration,  $R$  is the gas constant, and  $T$  is the absolute temperature. Also, for one osmol

$$v = \frac{1}{c} \quad \text{and} \quad dv = -\frac{1}{c^2} dc,$$

therefore

$$dE = -p dv = cRT \frac{1}{c^2} dc = RT \frac{dc}{c}.$$

The amount of energy required for the reversible transfer of one mol of substance from a solution of constant concentration  $a$  to concentration  $b$  will then be<sup>2</sup>

$$E = \int_a^b RT \frac{dc}{c} = RT \int_a^b \frac{dc}{c} = RT \ln \frac{b}{a}, \quad (1)$$

where  $\ln$  is the logarithm to the base  $e$ .

<sup>1</sup> Clearly, one liter of urine is not derived from one liter of glomerular filtrate in fact. However, since the process considered here and the actual process both accomplish the formation of urine from a solution having the composition of extracellular fluid, the hypothetical process is a valid basis for the equations, regardless of the enormous volume of the actual glomerular filtrate.

<sup>2</sup> Two very large vessels at temperature  $T$ , with regions permeable only to the solute in question are imagined. This solute is allowed to diffuse reversibly into a cylinder

The energy needed to transfer an infinitesimal amount of substance,  $dz_i$ , from a reservoir of constant concentration  $c_i$  with respect to the  $i$ th component to a one liter volume of concentration  $z_i$  will be given, according to equation (1) above, as

$$dE = RT \ln \frac{z_i}{c_i} dz_i.$$

Integration of this between the limits  $c_i$  and  $x_i$ , the urinary concentration of the  $i$ th component, will correspond to the energy, with regard to the  $i$ th component, required to form one liter of urine:<sup>3</sup>

$$\begin{aligned} E_i &= \int_{c_i}^{x_i} RT \ln \frac{z_i}{c_i} dz_i = c_i RT \int \ln \frac{z_i}{c_i} d\left(\frac{z_i}{c_i}\right) \\ &= c_i RT \left[ \frac{z_i}{c_i} \left( \ln \frac{z_i}{c_i} - 1 \right) \right]_{\frac{z_i}{c_i}=c_i}^{\frac{z_i}{c_i}=x_i} \\ &= RT \left( x_i \ln \frac{x_i}{c_i} + c_i - x_i \right). \quad (2) \end{aligned}$$

If there are  $n$  liters of urine in a given time, and  $y_i = nx_i$  is the corresponding total number of osmols of the  $i$ th component, the total amount of work will be, for that component,

$$\begin{aligned} nE_i &= nRT \left( x_i \ln \frac{x_i}{c_i} + c_i - x_i \right) \\ &= RT y_i \left( \ln \frac{x_i}{c_i} + \frac{c_i}{x_i} - 1 \right) \quad (3a) \end{aligned}$$

or

$$\begin{aligned} nE_i &= nRT \left( x_i \ln \frac{x_i}{c_i} + c_i - x_i \right) \\ &= RT \left( y_i \ln \frac{y_i}{nc_i} + nc_i - y_i \right) \\ &= RT \left( y_i \ln \frac{y_i}{c_i} - y_i \ln n + nc_i - y_i \right). \quad (3b) \end{aligned}$$

The total work  $E_T$  done by the kidney will be the sum of the energies for the individual com-

ponents:

$$\begin{aligned} E_T &= \sum_i nE_i \\ &= \sum_i RT \left( y_i \ln \frac{y_i}{c_i} - y_i \ln n + nc_i - y_i \right) \\ &= RT \left( \sum_i y_i \ln \frac{y_i}{c_i} \right. \\ &\quad \left. - \ln n \sum_i y_i + n \sum_i c_i - \sum_i y_i \right). \quad (4) \end{aligned}$$

These equations hold accurately only for dilute solutions; for more concentrated solutions, the concentrations must be replaced by activities. This would, however, obscure the simplicity of the results, and in many cases, sufficient data are not at hand to make this change. Quantitative results of these equations will be only slightly inaccurate, and for most biological purposes may be considered valid.

The values of the energy given above are the values required for the idealized, thermodynamically reversible formation of urine, and thus represent the useful work done by the kidney, rather than the actual energy it must expend. Both inefficiency in the actual performance of the osmotic work, and simple back diffusion, will reduce the overall efficiency of the tubules.

#### ON THE REDUCTION OF OSMOTIC WORK

A time-honored form of treatment for any diseased organ is the reduction of the work that must be done by that organ. Applied to the kidney this implies the reduction of the osmotic work. Equation (4) above should thus be examined with the view of making  $E_T$  as small as possible. One important and easily controlled variable is the total volume  $n$  of the urine, determined largely by the amount of water drunk. Considering the rate of excretion  $y_i$  of the various components, and also the composition of the glomerular filtrate as fixed, we may find the value of  $n$  to make  $E_T$  a minimum by setting

$$\frac{\partial E_T}{\partial n} = 0.$$

From equation (4) this gives

$$\frac{\partial E_T}{\partial n} = RT \left( -\frac{1}{n} \sum_i y_i + \sum_i c_i \right) = 0,$$

from the first vessel at partial pressure  $a$ ; a piston then compresses the substance to pressure  $b$ , and it is then allowed to diffuse into the other vessel at a pressure  $b$ . This is a simple application of one of the ideas involved in the "equilibrium box." A discussion of this and van't Hoff's law may be found in texts on physical chemistry, for example, Glasstone's "Text-Book of Physical Chemistry."

<sup>3</sup> See, for example, Dwight's "Table of Integrals."

whence

$$\sum_i c_i = \frac{1}{n} \sum_i y_i = \sum_i x_i. \quad (5)$$

Thus we see that to make the total work the least, the amount of water should be adjusted so that the total osmolar concentration of the urine ( $\sum x_i$ ) equals the total osmolar concentration of the glomerular filtrate ( $\sum c_i$ ). For a human urine of ordinary composition, the work is plotted as a function of the total daily volume in Figure 1, curve A. The minimal value is seen to be at  $n = 2.36$  liters, in agreement with equation (5). Although urea is the main urinary constituent,  $E_T$  has its minimum at a point where the ratio of  $x_{\text{urea}}$  to  $c_{\text{urea}}$  is still large, due to the fact that there are constituents with a ratio  $\frac{x_i}{c_i}$  much less than 1 (almost 0 for glucose), and for these the work increases with increasing  $n$ .

It is interesting to note that the usual urine volume is not far from the volume which results in the minimal work. Due to the small slope of the curve in the region of the minimum, the

amount of work associated with the ordinary urine volume is even more strikingly close to the minimal work.

Although not as easily subjected to control as the volume  $n$ , changes in the rate of excretion of the solid components are important. Two cases may be distinguished, one in which  $n$  is constant and only the amount of one solid changes, and the other in which  $n$  is always adjusted so as to be at the optimal value. The first case is solved directly from equation (4), and the second case from equation (4) after the value of  $n$  given by equation (5) has been substituted in (4). Fixing our attention on the  $j$ th component, we want to determine  $y_j$  so as to make  $E_T$  a minimum, and so we set

$$\frac{\partial E_T}{\partial y_j} = 0.$$

In the first case this gives

$$\frac{\partial E_T}{\partial y_j} = RT \left( y_j \frac{c_j}{y_j c_j} + \ln \frac{y_j}{c_j} - \ln n - 1 \right) = 0,$$

therefore

$$\ln \frac{y_j}{c_j} = \ln n$$

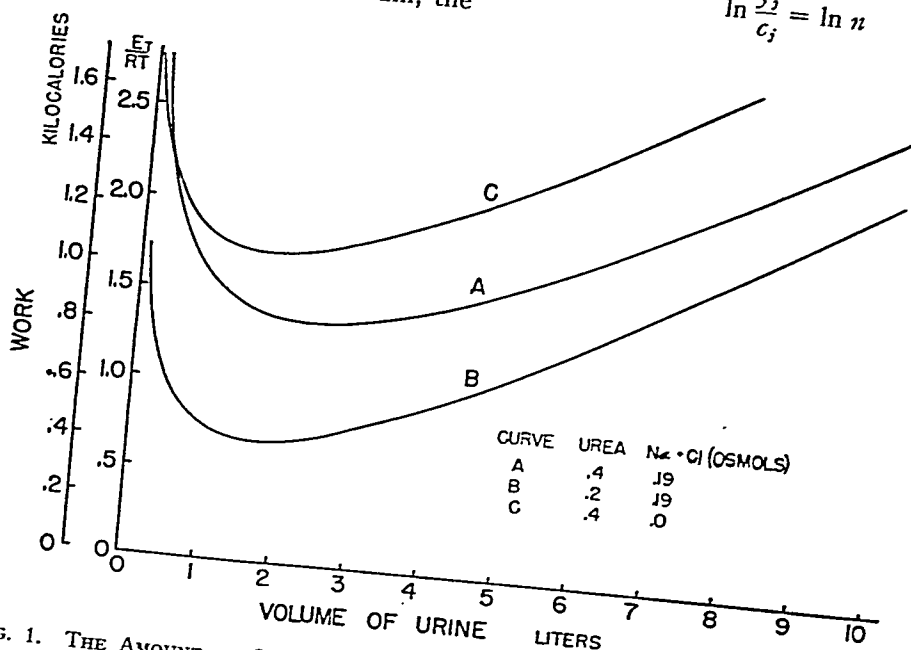


FIG. 1. THE AMOUNT OF OSMOTIC WORK REQUIRED FOR THE FORMATION OF URINE, PLOTTED AS A FUNCTION OF TOTAL DAILY URINE VOLUME

Curve A is based on a typical normal urine of a subject on a high-protein diet. Curve B is based on a urine containing only  $\frac{1}{2}$  as much urea as that of curve A, but the same in other respects. Curve C is calculated for a urine differing from that of curve A in that it is NaCl free.

or

$$\frac{y_j}{c_j} = n \quad \text{and} \quad x_j = \frac{y_j}{n} = c_j.$$

In the second case we have

$$\frac{\partial E_T}{\partial y_j} = RT \left( y_j \frac{c_j}{y_j c_j} + \ln \frac{y_j}{c_j} - \ln \frac{\sum_i y_i}{\sum_i c_i} - \sum_i y_i \frac{\sum_i c_i}{\sum_i y_i \sum_i c_i} \frac{1}{c_i} \right) = 0,$$

therefore

$$\ln \frac{y_j}{c_j} = \ln \frac{\sum_i y_i}{\sum_i c_i}$$

or

$$\frac{y_j}{c_j} = \frac{\sum_i y_i}{\sum_i c_i} = n \quad \text{and again} \quad x_j = c_j.$$

In both cases, we thus reach the obvious conclusion that  $E_T$  is a minimum when the urinary concentration  $x_j$  equals the concentration in the glomerular filtrate  $c_j$ . It may be stated more generally, that  $E_T$  will be reduced if  $x_j$  is changed in the direction which makes it more nearly equal to  $c_j$ .

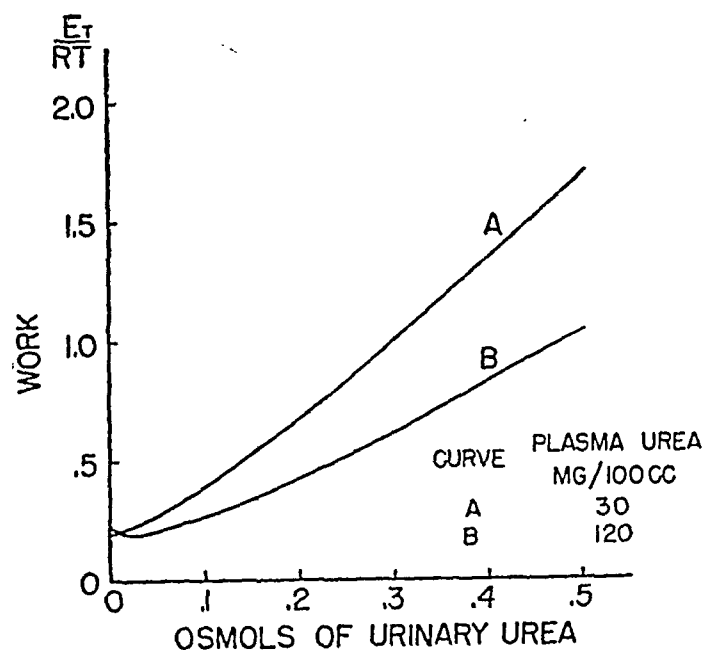


FIG. 2. CURVES WHICH SHOW HOW THE WORK IS INFLUENCED BY THE AMOUNT OF UREA EXCRETED DAILY

For each point the amount of water has been adjusted so as to give minimal work (equation (5)). Curve A is for a normal value of the plasma urea, while it is elevated to four times this value in curve B.

These results are important when applied to urea. Since urea is always greatly concentrated in the urine, a reduction in the amount of urea excreted will reduce the work. Curve A of Figure 1 is drawn for 0.4 osmoles (24 grams) of urea. Curve B represents a urine differing from that of curve A only in that it contains 0.2 osmoles (12 grams) of urea per day. It is seen that a reduction of urea causes a significant reduction in the amount of work. In Figure 2, curve A also shows the effect on the energy of changing urinary urea. For each point, the amount of water is assumed to be that which will give the minimal amount of total work.

The experiments of Addis (2) show that rats, whose kidneys have been reduced to  $\frac{1}{4}$  normal size, improve on a low-protein diet but become much worse on a high-protein diet. Since the rats on the high-protein diet excreted much more urea than the others, their kidneys did more osmotic work. This is interpreted as the basis for the marked difference in response of the two groups.<sup>4</sup>

When the generalization given above is applied to a substance less concentrated in the urine than in the glomerular filtrate, it follows that there will be a reduction in the work if the urinary concentration of the substance is increased. For example, the kidney must do work to prevent the excretion of glucose, and less work would be done if glucose were excreted so that the urinary and plasma concentrations were equal. Similarly, if the urinary concentration of NaCl is the same as the plasma concentration, no work is involved in its excretion, but if the dietary NaCl is limited, so that the urinary concentration is low, the kidney must do an increased amount of work. In Figure 1, curve A is drawn for  $\text{Na} + \text{Cl} = 0.19$  mol per day, whereas curve C is the same, except NaCl is no longer being excreted. Curve C is seen to represent more work, especially for dilute urines.

Of the variables determining  $E_T$  in equation (4), we have considered  $n$  (total volume) and  $y_i$  (amounts excreted);  $c_i$  (plasma concentrations) remain to be considered. Examination of  $\frac{\partial E_T}{\partial c_j}$  shows that as  $c_j$  approaches the corresponding

<sup>4</sup> It should be noted that some high-protein diets have a specific harmful effect on the kidneys (4).

urinary concentration  $x_j$ , the work  $E_T$  is reduced.

For substance with  $\frac{x_j}{c_j}$  less than 1 (e.g. glucose),

decrease of  $c_j$  will reduce the work, while if  $\frac{x_j}{c_j}$  is greater than 1, increasing  $c_j$  will decrease the work. An increase in the blood urea above the normal value will cause a decrease in the work. In Figure 2, curve A is drawn for a plasma urea of 0.005 osmols per liter (30 mgm. urea per 100 cc.) and curve B is for 0.020 osmols per liter (120 mgm. urea per 100 cc.). Curve B always is under curve A, except for the trivial case of urines with very low urea contents.

#### THE RESULTS OF LIMITATION OF OSMOTIC WORK

When the amount of osmotic work being done by the kidney is limited by the capabilities of the kidney, the results of this limitation may often be predicted from the equations for osmotic work. This limitation may occur in a normal kidney when an unusually large osmotic demand is made upon it, due, for example, to the restriction of drinking water. On the other hand, the kidney may be so injured that even the normal amount of work cannot be done. Thus, in severe chronic nephritis, many changes are observed which, as we shall see, may be referred to lack of ability to perform osmotic work. The result of limiting  $E$  in the formulas for osmotic work is postulated to be the same as the changes seen in certain clinical conditions, notably severe chronic nephritis. This appears to be a reasonable postulate, and further support is lent it by the agreement of predictions based on it with fact.

It appears from Figure 1, or equation (4), that, for a urine containing any definite amount of solids per unit time, for each value of the work, there are two values of the volume  $n$ , corresponding to two concentrations. For values of  $n$  between these two, the energy will be less, but for values of  $n$  outside of this range, the energy will be greater. Thus, if the kidney is restricted to a certain maximum work, the concentration range of the urine is determined. As the maximum work decreases, the range of concentrations becomes narrower, so that for very restricted energies, the concentration has a very narrow range, corresponding to the minimal region of

the curve. Since the minimum of the curve is at the point where the total osmolar concentration of the urine equals that of the glomerular filtrate (equation (5)), extreme limitation of the work implies an osmolar concentration of the urine not far from that of the glomerular filtrate, no matter what solids the urine contains.

In concentration-dilution tests, the range of possible concentrations is measured. As the urea clearance falls, the range of concentration becomes narrower (5). For extremely impaired renal function one would expect a specific gravity of about 1.010, corresponding to an osmolar concentration equal to that of the plasma. For very low urea clearances, the range of specific gravities is about 1.008 to 1.013 (from Alving and Van Slyke, Figure 3 (5)). These facts are in agreement with the theoretical predictions.

Besides these limitations, restrictions in the energy which can be expended with regard to single components is to be expected. The consequences of this limitation may be seen conveniently from equation (2). Figure 3 is a graph of this function, for  $c = 0.1$  and  $c = 0.2$ . There is a zero value of the energy when  $x = c$ , the energy rising both for more dilute and more concentrated urines. For dilute urines, the slope of the curve is negative, so that the energy decreases with increasing concentration, while for urines concentrated with regard to the component under consideration, the energy increases with increasing concentration. There are thus two cases to be considered, one in which  $x < c$ , the other with  $x > c$ .

For a urine with a concentration greater than that of the glomerular filtrate with respect to a given component, a reduction in the energy must cause a lessened urinary excretion of the substance, and retention results. Retention will occur until a new blood concentration is reached, which is high enough so that the required amount of substance can be removed with the limited energy. For example, in Figure 3, normally 0.5 osmols per liter are removed by expenditure of energy  $E = .405RT$  calories per liter, with the normal plasma concentration of  $c = 0.1$ . The possible energy expenditure is then reduced to  $E = .158RT$  and retention occurs because  $x$  is reduced to 0.32, until the plasma concentration reaches  $c = 0.2$ . At this elevated plasma level,

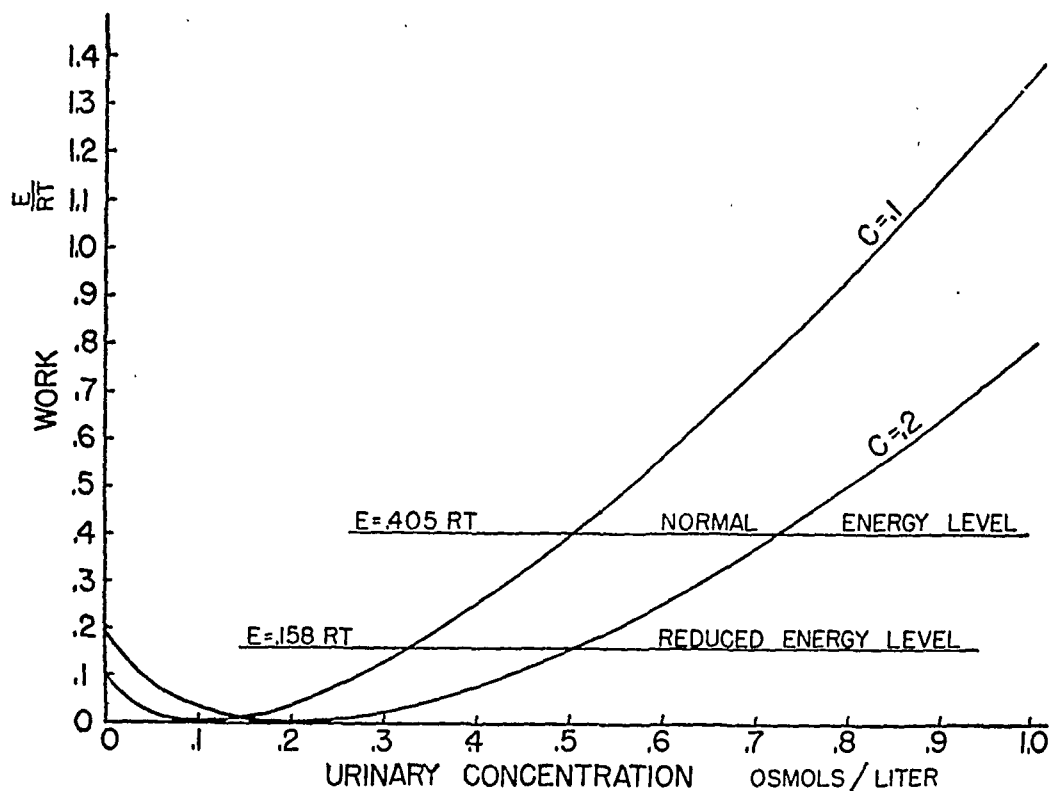


FIG. 3. ILLUSTRATING, FOR A HYPOTHETICAL EXAMPLE, HOW REDUCTION OF RENAL FUNCTION WILL CAUSE PROGRESSIVE RETENTION UNTIL THE PLASMA LEVEL HAS BECOME HIGH ENOUGH SO THAT, EVEN WITH THE REDUCED ABILITY TO DO WORK, SUFFICIENT MATERIAL CAN BE EXCRETED

It is also apparent that for a substance falling to the left of the minimum, reduction of renal function will cause an increased excretion of the substance.

the normal 0.5 osmols per liter can be removed with the limited energy, and no further retention occurs. A high blood urea may thus be considered both a result of a subnormal kidney, and a compensation for it.

Retention of urea, phosphate, and sulfate, all of which are greatly concentrated in the urine, occurs commonly in cases of kidney disease. Retention of urea occurs also after deprivation of water (6). Poisoning of the tubules with NaCN likewise causes reduced excretion of creatinine in dogs (7).

For a substance with respect to which the urine is relatively dilute, a reduction in the osmotic work necessitates an increase in urinary concentration, as may be seen from the left-hand part of the curves in Figure 3. Unless there is compensation for this increased excretion, the plasma level of the substance will fall until the rate of excretion is reduced to its normal value. Thus, when the intake of NaCl is not large, the plasma has a higher NaCl concentration than does the urine. Limitation of the osmotic work

may thus result in an increased excretion of NaCl, and a lowered plasma level.

In severe chronic nephritis, the expectation of a reduced plasma concentration of Na, accompanied by an increase of plasma phosphate, sulfate, and urea, is actually realized (8). Also, kidneys poisoned with NaCN excrete more glucose and chloride, as shown in isolated dog kidneys (7).

If the energy expended on a substance is limited to a constant maximal value, and the substance is being excreted at a constant rate, it follows from equation (3a) that the ratio  $\frac{x}{c}$  is constant. If the volume of urine is increased, there will be a temporary increase in the amount of substance excreted, until  $c$  falls so that the ratio  $\frac{x}{c}$  has reached its former value, and then  $c$  will fall no more. This corresponds with the fact that on increasing the water drunk, the high blood urea of patients with chronic nephritis falls to a lower level, which is still above normal, and

then remains constant at this lower level. The augmentation limit found in normal subjects may represent a similar, much higher, limitation on the energy expenditure.

For NaCl, when its dietary intake is restricted, an increase in the urinary volume must either result in an increased expenditure of energy, or a reduction of the plasma level so that the previous  $\frac{x}{c}$  ratio is re-established. If the energy is limited, a reduction of the blood  $\text{Na}^+$  is to be expected. In the treatment of nephritic edema, removal of body Na is desired. A restricted intake of Na and a large urine volume would appear to work toward this end. The clinical effectiveness of this method has been shown (9). This procedure produces an increase in the osmotic demand made on the kidney, but is justified in this case because of its special therapeutic effect, the removal of edema. When edema is not an issue, there is no reason for not adjusting water and NaCl so as to give the minimal amount of work, and this means giving suitably large amounts of NaCl.

Sweat is a solution containing a lower concentration of NaCl than the plasma. An increase in the rate of production of sweat at a given concentration will require an increased rate of work by the sweat glands. If this work cannot be done, the concentration of NaCl in the sweat must increase. It has been found that when sweating is very profuse, the concentration of NaCl is elevated (10).

Thus far we have considered only the work required for the idealized formation of urine. Although a satisfactory interpretation of many phenomena can be given on the basis of this simple idea, it would not be surprising if a more realistic consideration were sometimes required. The following example may be such a case, although an alternative interpretation is possible. Postulation of an upper limit to the rate at which the kidney can do osmotic work in reabsorbing glucose gives rise to a "threshold." But this cannot account for the fact that with high values of the blood glucose, the urinary concentration exceeds the blood concentration, since less osmotic work would be done if less glucose were excreted. This may result from the fact that, in order to make a substance pass through the

tubular wall at a finite rate, work must be done, due to viscosity, aside from the osmotic work, due to a concentration difference. This effect is, of course, not taken into account in the equation for the reversible work. That glucosuria is due to the inability of the kidney to do osmotic work may be questioned. If another mechanism is involved, there would be no contradiction with even the simpler consideration.

In the case of substances which are more dilute in the urine than in the plasma, consideration of the work required for the diffusion through the tubular wall produces no contradiction, because limitation of either this work or the osmotic work requires the excretion of more of the substance. Furthermore, it may be inferred from the observed facts discussed above that in many cases the osmotic work is the determinative factor.

#### SUMMARY

The equation which gives the amount of work required for the idealized formation of urine has been examined in order to determine the amounts of excretory water and solids which will give the least work. Furthermore, the effect of changes in blood concentrations on osmotic work is determined. The changes necessitated by limitation in osmotic work are predicted and examples are given where these predictions agree with clinical findings. These considerations suggest certain therapeutic procedures, whose validity can be determined only by actual trial.

I am grateful to Doctors J. L. Gamble and L. H. Newburgh for encouragement throughout the preparation of this paper.

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# DIODRAST AND INULIN CLEARANCES IN NEPHROTIC CHILDREN WITH SUPERNORMAL UREA CLEARANCES

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(Received for publication December 31, 1942)

Elevation of the urea clearance above the calculated normal value has been frequently observed in our clinic in children with the nephrotic syndrome. Previous studies of this phenomenon have shown it to be related to the intake of protein, but not induced by the oral administration of urea (1). Glomerular filtration, measured by inulin clearance, has been found by Emerson, Fletcher, and Farr (2) to be elevated as much or more than the urea clearance. The present study is an attempt to answer the question raised in the last report (2) as to whether renal blood flow is elevated proportionately to the glomerular filtration rate. Evidence presented by Goldring, Chasis, Ranges, and Smith (3) and by White and Heinbecker (4) indicates that the diodrast clearance approximates the renal blood flow in man. In order to obtain some indication as to whether the high urea and inulin clearances observed in nephrosis are probably due to filtration of an increased fraction of plasma water or to an increased renal blood flow, we have therefore, in 4 cases, made a series of simultaneous determinations of the clearances of urea, inulin, and diodrast.

## MATERIAL

Four nephrotic children, aged 3, 5, 5, and 10 years, were selected for study, the oldest of whom (R.Q.) had also been included in the previous group (2). All had been maintained during at least the preceding 4 months on diets high in protein (over 3 grams per kgm. per day), and low in salt (less than 1.6 grams per day). Routine urea clearances during this period showed values around 140 per cent of normal or higher.

## METHODS

Urea was determined by the hypobromite method (5), diodrast by colorimetric estimation of liberated iodine (6), and inulin by measurement of the color developed with diphenylamine reagent (7, 8). For colorimetric measurements, a Klett-Summerson colorimeter was used.

As in previous work, clearance values are corrected for differences in size of the subjects by applying the surface area factor of Møller, McIntosh, and Van Slyke (9) to the urine flow figures. In the instances of corrected urine flows lower than 2 cc. per minute, maximal plasma clearances of urea were calculated by the formula:

$$\text{Maximal clearance} = \frac{U \sqrt{2V_c}}{P},$$

where  $U$  is urine urea concentration,  $P$  is plasma urea concentration, and  $V_c$  is the corrected urine volume in cc. per minute (see (2) p. 364). Normal maximum plasma clearance is taken as 72 cc. per minute per 1.73 sq. M. for the purpose of calculating percentages. This figure is derived from the usual standard of 75 cc. per minute per 1.73 sq. M. for whole blood urea clearance (9) by assuming that plasma urea concentration is 4 per cent higher than whole blood concentration.

## PROCEDURE

Five experiments were performed on the 4 subjects. In each experiment, continuous urea clearances were measured during the preceding 2 to 5 hours in order to observe any variations that might be induced by subsequent manipulations. Water was given hourly in 100 cc. portions, beginning 3 hours before the test. Priming doses of 2 to 4 grams of inulin and 1 to 2 cc. of a 35 per cent diodrast solution, depending on the size of the subject, were given in 100 cc. of saline, intravenously, over a period of about 10 minutes, followed by continuous infusion of a solution which contained 1 to 2.5 per cent inulin and 0.6 to 2.0 per cent of 35 per cent diodrast. This was given at a rate of approximately 3 cc. per minute throughout the experiment. No measurements of inulin or diodrast clearances during the initial 10 to 30 minutes of equilibration are included, although urea clearances were continued during this time.

## DISCUSSION

The distribution of observed clearance values in relation to normal adult standards is shown on a log-log graph (Figure 1), with urea clearance values as abscissae, and clearances of diodrast and inulin as ordinates. This type of graph is used because, if the clearance ratios,

$$r_1 = \frac{\text{urea clearance}}{\text{diodrast clearance}}$$

and

$$r_2 = \frac{\text{urea clearance}}{\text{inulin clearance}},$$

are constant, the values of their denominators fall on straight lines with slopes of 45°; it is thus

possible to make a visual estimate of the variability of clearance ratios as well as of the deviation of the clearance values themselves from normal standards. The areas indicating the normal ranges of the ratios are plotted from data

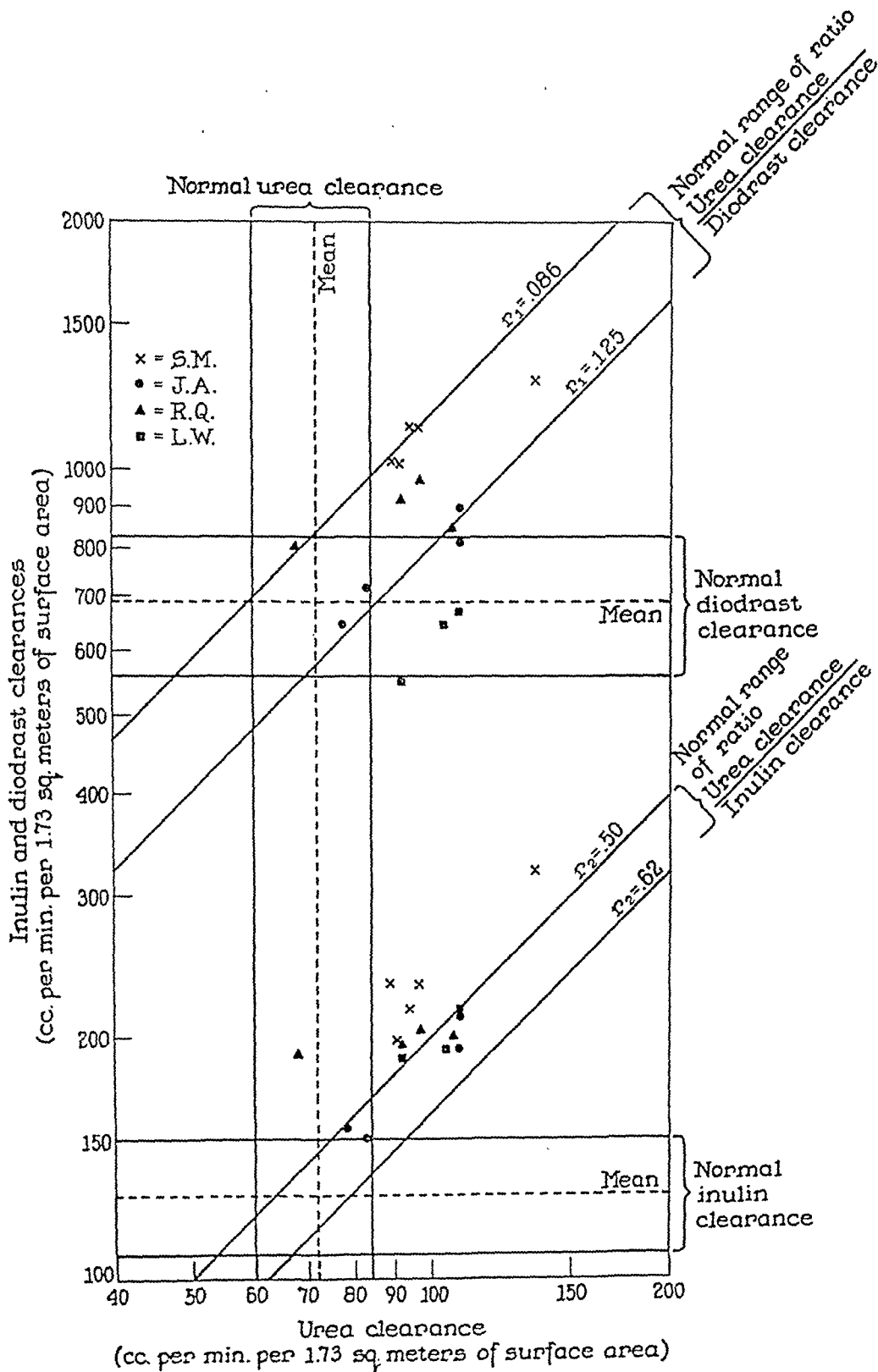


FIG. 1. LOG-LOG GRAPH OF THE INULIN AND DIODRAST CLEARANCES, PLOTTED AGAINST THE UREA CLEARANCE OF 4 CHILDREN WITH NEPHROSIS

TABLE I  
Summary of clearance data

Subject	Period	Duration	V <sub>e</sub>	Plasma			Urine			Clearances				Ratios			
				U	I	D	U	I	D	U		I	D	U/I	I/D		
										$\frac{UV_e}{P}$	Per cent normal	$\frac{UV_e}{P}$	$\frac{UV_e}{P}$				
March 22, 1942 J. A. ♀ 5 years factor = 2.52	1 2 3 4 5 6	96 25 31 29 31 20	6.19 7.16 3.62 8.08 3.90 5.29	8.1			102 111 185 109 161 165			102 3000 1280 1940 1610 285	78 99 83 109 78 108	108 136 115 151 108 149		150 714 194 812 154 641 898	0.57 0.57 0.52 0.52	0.21 0.24 0.24 0.24	
Average											92	128	178	766	0.55	0.23	
March 12, 1942 L. W. ♀ 3 years factor = 2.64	1 2 3 4 5 6	45 58 61 33 32 26	3.47 4.11 1.98 7.50 4.35 5.75	10.5			337 162 472 146 223 196			337 162 472 872 1506 1059	250 408 326	115 63 89 104 92 108	159 88 123 145 128 149		194 647 188 550 217 670	0.56 0.51 0.52	0.30 0.34 0.32
Average											95	132	200	622	0.53	0.32	
March 16, 1942 S. M. ♂ 5 years factor = 2.35	1 2 3 4 5	53 50 30 27 31	3.34 3.10 3.83 2.61 6.00	8.3			235 255 194 290 130			235 1400 1738 869 260	352 468 260	94 95 89 91 94	131 132 124 126 130		233 1006 197 1002 217 1130	0.40 0.48 0.45	0.22 0.16 0.19
Average											93	129	216	1046	0.44	0.19	
March 30, 1942 S. M.	1 2 3 4 5	250 58 37 38 29	0.99 1.17 0.93 2.35 6.50	7.1			760 572 736 406			760 5240 2330 778 158	880 386 158	109 94 97 135	210 172 197 187		232 1136 322 1277 253 1284	0.44 0.44	0.20 0.25 0.20
Average											109	192	270	1232	0.44	0.22	
R. Q. ♀ 10 years factor = 1.76	1 2 3 4 5 6	84 65 29 33 28 31	6.58 9.20 10.36 5.40 10.66 6.43	8.5 8.3 8.1 7.9 7.8 7.6			117 92 83 100 71 109			117 820 1300 740 1160 174	80 166 120	90 102 106 68 97 92	125 141 142 95 135 128		200 846 190 800 202 918	0.55 0.37 0.50 0.49	0.24 0.24 0.21 0.21
Average											92	128	197	884	0.48	0.23	

V<sub>e</sub> gives values of urine flow corrected to a surface area of 1.73 sq. M.; to obtain observed flows, divide by the factor given in the first column. U, I, and D represent urea, inulin, and diodrast, respectively.

obtained from adults (3), since the necessary data from normal children are lacking. This use of adult standards for comparison with results from children is partially justified by the fact that urea clearance values in children, when corrected to a surface area of 1.73 sq. M., fall into the same range as adult values, similarly corrected (10). There is a fair degree of presumption, therefore, that diodrast and inulin clearances per sq. M.

body surface may be the same for children as for adults, but this presumption remains to be verified by observations on normal children.

The mean normal plasma clearances, in cc. per minute per 1.73 sq. M. of body surface, have been found to be 72 for urea (with urine flows above 2 cc. per minute per 1.73 sq. M. surface), 125 for inulin (3), and 690 for diodrast (3). Compared with these, in our 4 nephrotic cases, the urea

clearances were 128 to 192 per cent, the inulin 140 to 220 per cent, and the diodrast 95 to 185 per cent as great as the mean normal values. Since in our 4 high urea clearance nephrotic children, the elevation of the urea clearance was found to be associated with an elevation of both the inulin and diodrast clearances, we conclude that the elevation of the urea clearance is due principally to an increase in renal blood flow (Table I).

#### SUMMARY

The elevated urea clearance in 4 nephrotic children was found to be associated with increase of both inulin and diodrast clearances.

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# OBSERVATIONS ON THE PHYSIOLOGY AND BIOCHEMISTRY OF QUANTITATIVE BURNS

By WILLIAM W. L. GLENN,<sup>1</sup> JYTTE MUUS, AND CECIL K. DRINKER

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Early in 1941, two of the authors began a series of experiments upon burns, being convinced that the technique of lymph collection familiar to them might permit profitable examinations of the amount and characteristics of the exudate from burned tissue. This conception depends upon the fact that tissue fluid, wherever it has been collected, and lymph are identical in composition (Drinker and Yoffey (1)).

Glenn, Peterson, and Drinker (2) published a first paper describing a readily repeatable experimental burn made by immersing the paw of an anesthetized dog in very hot water. Many experiments have been carried out on dogs, particularly those concerned with the development of new methods of treatment. But for experiments requiring large amounts of lymph in order to carry through extensive biochemical work, larger animals were needed and calves have proved very satisfactory.

Several recent reviews give a summary of the chemical changes which have been found to occur in blood from patients and experimental animals suffering from burns (3). There is general agreement that one finds an increase in non-protein nitrogen, urea, and creatinine, and a decrease in total protein and carbon dioxide combining capacity. The changes in some of the electrolytes seem to be a little less consistent, though in general there is an increase in potassium, and a decrease in sodium and chloride. The often repeated claim that there is an increase in "polypeptide nitrogen" does not seem to be supported by any clear-cut evidence.

In the present investigation, it was hoped to distinguish between what might be termed primary and secondary effects of the burn. That is to say, we attempted to determine whether any of the compounds which eventually accumulated in the blood came directly from the burned area.

or whether they were released through damage to some other tissues. We assumed that lymph coming from the burned area would contain a higher concentration of substances resulting from tissue damage due to the burn than would lymph from another part of the body, or serum taken from the general circulation. On the other hand, substances of small molecular size which accumulate in the serum owing to impaired function of any of the organs would be present in the same concentration in the serum and in the lymph collected from different parts of the animal.

## 1. Lymphatic cannulation in the calf

Baum (4) wrote a copiously illustrated monograph on the lymphatics of calves and adult cattle. His plates, though numerous, do not prove very helpful in finding the vessels one wishes to cannulate in order to obtain lymph from burned legs. It was necessary to make subcutaneous injections of T-1824 in order to fill the lymphatics, and eventually cannulation of the lymphatic, indicated in Figure 1 (B) was found to give most of the lymph from the foreleg.

The important landmark to be sought in locating the foreleg lymphatic is the large superficial cervical lymph node (A, Figure 1). This node receives afferents not only from the skin and deep tissues of the foreleg, but also from the skin of the neck and upper part of the thorax. These are, however, but incidental suppliers of lymph compared to what comes from the foreleg. Furthermore, they are not reached by the hot water applied to the leg, and probably remain a constant but small factor in the lymph collected after burning. It is characteristic of lymph nodes all over the body that while many afferent vessels enter them through the capsule, the efferent flow is concentrated in a single vessel which leaves the hilus of the node and is generally large. This is the case in the calf, and by placing a cannula in

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clearances were 128 to 192 per cent, the inulin 140 to 220 per cent, and the diodrast 95 to 185 per cent as great as the mean normal values. Since in our 4 high urea clearance nephrotic children, the elevation of the urea clearance was found to be associated with an elevation of both the inulin and diodrast clearances, we conclude that the elevation of the urea clearance is due principally to an increase in renal blood flow (Table I).

#### SUMMARY

The elevated urea clearance in 4 nephrotic children was found to be associated with increase of both inulin and diodrast clearances.

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# OBSERVATIONS ON THE PHYSIOLOGY AND BIOCHEMISTRY OF QUANTITATIVE BURNS

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Early in 1941, two of the authors began a series of experiments upon burns, being convinced that the technique of lymph collection familiar to them might permit profitable examinations of the amount and characteristics of the exudate from burned tissue. This conception depends upon the fact that tissue fluid, wherever it has been collected, and lymph are identical in composition (Drinker and Yoffey (1)).

Glenn, Peterson, and Drinker (2) published a first paper describing a readily repeatable experimental burn made by immersing the paw of an anesthetized dog in very hot water. Many experiments have been carried out on dogs, particularly those concerned with the development of new methods of treatment. But for experiments requiring large amounts of lymph in order to carry through extensive biochemical work, larger animals were needed and calves have proved very satisfactory.

Several recent reviews give a summary of the chemical changes which have been found to occur in blood from patients and experimental animals suffering from burns (3). There is general agreement that one finds an increase in non-protein nitrogen, urea, and creatinine, and a decrease in total protein and carbon dioxide combining capacity. The changes in some of the electrolytes seem to be a little less consistent, though in general there is an increase in potassium, and a decrease in sodium and chloride. The often repeated claim that there is an increase in "polypeptide nitrogen" does not seem to be supported by any clear-cut evidence.

In the present investigation, it was hoped to distinguish between what might be termed primary and secondary effects of the burn. That is to say, we attempted to determine whether any of the compounds which eventually accumulated in the blood came directly from the burned area.

or whether they were released through damage to some other tissues. We assumed that lymph coming from the burned area would contain a higher concentration of substances resulting from tissue damage due to the burn than would lymph from another part of the body, or serum taken from the general circulation. On the other hand, substances of small molecular size which accumulate in the serum owing to impaired function of any of the organs would be present in the same concentration in the serum and in the lymph collected from different parts of the animal.

## 1. Lymphatic cannulation in the calf

Baum (4) wrote a copiously illustrated monograph on the lymphatics of calves and adult cattle. His plates, though numerous, do not prove very helpful in finding the vessels one wishes to cannulate in order to obtain lymph from burned legs. It was necessary to make subcutaneous injections of T-1824 in order to fill the lymphatics, and eventually cannulation of the lymphatic, indicated in Figure 1 (B) was found to give most of the lymph from the foreleg.

The important landmark to be sought in locating the foreleg lymphatic is the large superficial cervical lymph node (A, Figure 1). This node receives afferents not only from the skin and deep tissues of the foreleg, but also from the skin of the neck and upper part of the thorax. These are, however, but incidental suppliers of lymph compared to what comes from the foreleg. Furthermore, they are not reached by the hot water applied to the leg, and probably remain a constant but small factor in the lymph collected after burning. It is characteristic of lymph nodes all over the body that while many afferent vessels enter them through the capsule, the efferent flow is concentrated in a single vessel which leaves the hilus of the node and is generally large. This is the case in the calf, and by placing a cannula in

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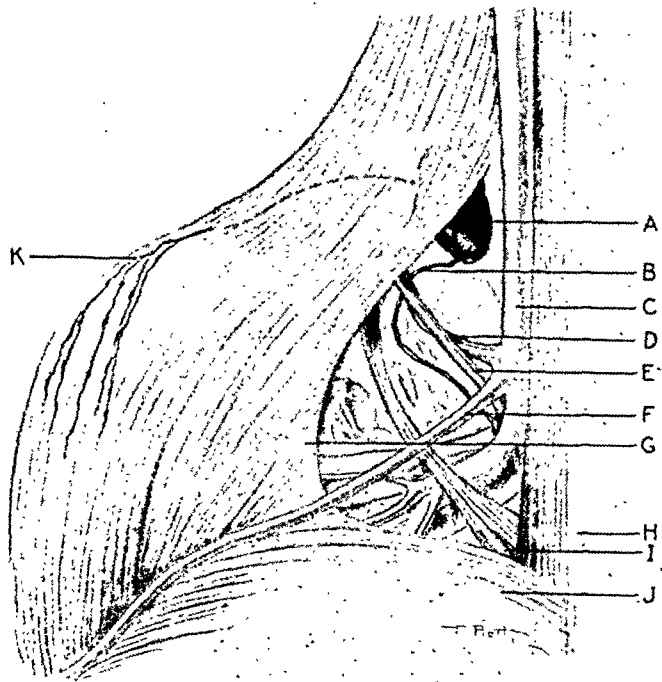


FIG. 1. SEMIDIAGRAMMATIC DRAWING OF THE LYMPHATIC USED IN COLLECTING LYMPH FROM THE FORELEG OF A CALF

In orienting the drawing, the head of the calf is toward the top of the drawing.

A, superficial cervical lymph node. B, efferent lymphatic running either directly into the common jugular vein or into the right lymphatic duct. This is the vessel cannulated to collect lymph. C, external jugular vein. D, inferior cervical vein. E, inferior cervical artery. F, cephalic vein. G, mastoido-humeralis muscle retracted to show lymph node. H, sterno-cephalicus muscle. I, relatively constant slip of the mastoido-humeralis muscle, a valuable landmark when present. J, superficial pectoral muscle. K, afferent lymphatics draining foreleg.

the efferent lymphatic (B, Figure 1), one obtains the large amounts of lymph required for analyses before and after burning.

## 2. Measures to promote lymph flow from burned tissues and the technique of quantitative burning, employing hot water

It is generally believed that lymphatics and blood vessels in a swollen part are compressed and cease to conduct fluid. Nothing could be further from the truth. In both cases, but especially in that of the lymphatics, the vessels are attached by fine bands of connective tissue to surrounding structures, and swelling results in dilatation rather than constriction. Pullinger and

Florey (5) showed this particularly well for the lymphatics in local edema.

In calves, the legs even if burned severely do not swell as dramatically as in the case of the dog. The skin is tougher and more tightly applied to the underlying muscles. In order, therefore, to obtain a copious and continuous flow of lymph, the anesthetized calf is arranged as is shown in Figure 2. This results in a steady flexion and extension of the forelegs as the  $\frac{1}{2}$ -horsepower motor (A), operating through reducing gears, rotates the crank (B) 14 times a minute. The crank is connected with the forehoofs by means of two sash cords (C) readily seen in the illustration. This constant uniform motion results in a large lymph flow, which falls off only as coagulation occurs widely through the burned region and extends into the lymphatics. The problem of producing a burn by means of hot water in a calf weighing in the neighborhood of 200 pounds is not entirely simple, especially when it is recollected that the application of the water should be to a precise level upon the leg of the animal, that the duration of the exposure be regulated accurately, and that the temperature of the water be held reasonably constant. We have accomplished these requirements quite satisfactorily by making use of a piece of 8-inch galvanized iron pipe, over the lower end of which a heavy sponge-rubber diaphragm reinforced by a thick sheet of solid rubber has been tied securely. This diaphragm is of the type used in an infant-size respirator and has a 2-inch hole in the center. With the animal upon his back, as shown in Figure 2, a front or hind hoof is thrust through the hole in the diaphragm and the galvanized container pushed centrally until the diaphragm has reached the selected upper limit of the prospective leg burn and a water-tight seal around the leg is obtained. The leg of the animal and the enclosing can are then securely suspended in a vertical position. Water heated to the required temperature is poured rapidly into the can and remains in contact with the leg as long as is desired. At the close of exposure, a large side vent just above the rubber diaphragm permits rapid emptying of the water.

In the present experiments, boiling water was used and it was left in contact with the leg for

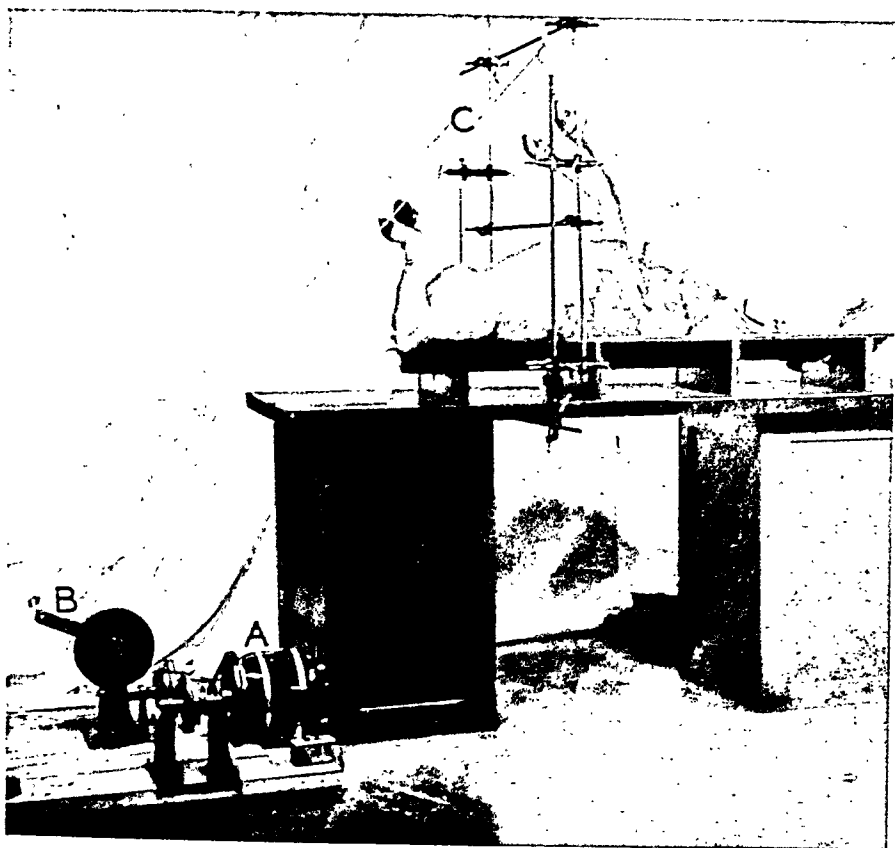


FIG. 2. CALF ANESTHETIZED WITH NEMBUTAL

Lower gauze pad is over the cannulated left foreleg lymphatic. Upper gauze pad covers the carotid artery cannulated for taking blood pressure and samples of arterial blood. The lymphatic for the right foreleg has been cannulated on the opposite side. The  $\frac{1}{2}$ -horsepower motor, *A*, through reducing gears, rotates the arm, *B*, 14 times a minute. Through the cords, *C*, fastened to the hoofs, this rotation results in rhythmic flexion and extension of the forelegs, and, through this passive motion, steady flow of lymph.

from  $1\frac{1}{2}$  to 4 minutes, the actual time in each experiment being recorded.

Lymph for analysis was collected simultaneously from both front legs by cannulation of the efferent lymphatic of the superficial cervical node for 2 to 3 hours before the animal was burned; then one leg was burned and collection was continued, keeping the lymph from the two legs separate and changing receivers at stated intervals, so that a series of samples representing different time intervals after the burn was at hand. One, and in some cases two, more extremities were burned after varying lengths of time.

Blood was taken before the burn, once or twice during the experiment, and at the end. The samples were usually drawn from the jugular vein,

except when the animal died before the termination of the experiment, in which case the final blood was taken from the heart.

The calves used have weighed from 125 to 200 pounds, and were weaned some time previously. Calves still upon milk or just weaned arrive at the slaughter house in very variable states of hydration and nutrition. Older animals are in much more stable condition, but even so are given 10 cc. of Ringer's solution per pound of weight, intraperitoneally or intravenously, so that they may have a ready supply of fluid to draw upon. It has been our practice to anesthetize these animals at the slaughter house, using 2.5 per cent nembutal in the amount of 1 cc. for 4 pounds of body weight. This is given intravenously and very

slowly, stopping the injection from time to time and often finishing short of the full dose, depending upon the condition, particularly the respiration of the animal. Even with this small dose, about half that used for dogs, we have lost two animals at the outset of the experiment. Once anesthesia is well established, the chances of disaster from the anesthetic are very much less.

### 3. Chemical methods

The study was confined to some of the nitrogenous compounds always present in normal blood.

Total protein was usually determined with the Zeiss Eintauch-Refraktometer (Pulfrich), and the values were frequently checked by micro-Kjeldahl. In the micro-Kjeldahl, the ammonia was steam-distilled and titrated in 2 per cent boric acid with methyl red as indicator. The method is a modification of Wagner's (6). The digestion was done either by the method of Wong (7) or by a slight modification of the method of Schwoegler, Babler, and Hurd (8).

The same micro-Kjeldahl procedure was used to determine the non-protein nitrogen in the tungstic acid filtrate from serum or lymph prepared according to Haden's modification of the Folin-Wu method (7). Creatinine and creatine + creatinine were done on the tungstic acid filtrate according to Folin (9). Amino-nitrogen was determined by the gasometric method of Van Slyke (7) on whole serum and lymph and on the tungstic acid filtrate. Urea was determined on whole serum and lymph by the gasometric method of Van Slyke (7).

Oxygen content and oxygen combining capacity were determined according to Van Slyke (7).

### 4. The flow and protein content of lymph from the normal and burned leg of the calf

We have been unable to find data upon the flow and composition of lymph even from the thoracic duct of cattle. Our experience with 16 calves thus supplies information upon normal conditions as well as the contrast which occurs following a severe burn. Table I is a summary of fundamental data upon 4 animals. In all cases, the lymph flow from the burned leg increased markedly immediately after the burn, and at the same time, there was an increase in the total protein of from 35 to 75 per cent of the original values. The steady decrease in the rate of lymph flow during the course of the experiment is not accompanied by a decrease in protein content, which stays very constant at the high level established right after the burn. A detailed study on the distribution of the different proteins in the lymph and serum is reported elsewhere (10).

As in the case of dogs, with which we have had prolonged experience, there is no correlation between the amount of lymph flowing from a cannulated vessel and the weight of the animal. Notice also that the largest percentile increases in lymph production as a result of burning occur when the normal flow of lymph is comparatively small. In all of these animals, the uniform passive motion described in a previous section was

TABLE I

*The lymph flow and protein content from the normal and burned forelegs of 4 calves, per hour for consecutive hours immediately before and after burn*

Number of calf	Weight	Control blood protein	Forelegs prior to burning				Forelegs following burning*			
			Lymph flow		Lymph protein		Lymph flow		Lymph protein	
			R	L	R	L	R	L	R	L
			<i>cc. per hr.</i>		<i>per cent</i>		<i>cc. per hr.</i>		<i>per cent</i>	
2	200	6.0	7.4 7.3	4.7 4.2	2.8	2.2	12.9 8.0	48.3 41.0	3.1	3.7
6	165	5.4	7.0 6.5	9.0 6.0	2.5	2.6	25.5 28.0	9.2 6.0	4.4	2.6
8	155	6.7	20.0 21.0	23.0 22.0	3.1	3.1	12.0 10.0 8.5	36.0 15.0 19.0	3.1	4.3
9	190	6.3	21.5 18.0 14.5	22.0 19.0 11.0	2.9	2.9	23.0 33.0 27.0	17.0 19.0 11.0	3.8	2.7

\* Burned forelegs in italics.

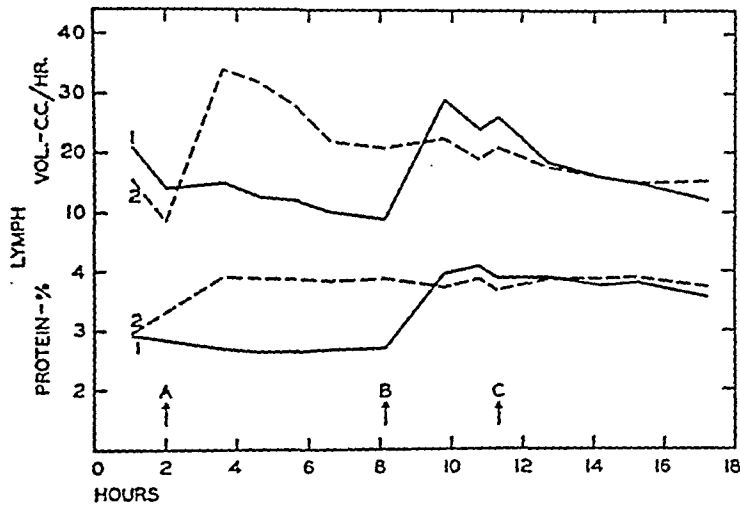


FIG. 3. CURVES SHOWING THE RATE OF FLOW AND PROTEIN CONTENT OF LYMPH FROM THE NORMAL AND BURNED FORELEGS OF A CALF WEIGHING 170 POUNDS

Ordinates, protein in per cent and lymph flow per hour in cubic centimeters. Curve 1 (solid line, upper), cubic centimeters of lymph per hour from right foreleg. From 0 to arrow B, normal period. At arrow B, immersed 3 minutes in water at 100° C. Curve 1 (solid line, lower), protein concentration in per cent of right foreleg lymph. Curve 2 (broken line, upper), cubic centimeters of lymph per hour from left foreleg. From 0 to arrow A, normal period. At arrow A, immersed 3 minutes in water at 100° C. Curve 2 (broken line, lower), protein concentration in per cent of left foreleg lymph. At arrow C, the left hind leg was immersed in water at 100° C. for 3 minutes.

in operation during the period of normal collection and after burning. Lacking this mild cause of lymph movement, no lymph would be secured during the control period, and though a substantial flow would follow burning, it would be much less than that accompanying constant passive motion.

Figure 3 presents a continuous record of lymph flow and lymph protein from a calf weighing 170 pounds and severely burned. At arrow A, the left foreleg was immersed in water at 100° C. for 3 minutes. The effects on lymph flow and lymph protein content are shown in the broken lines, 2 and 2. At arrow B, the right foreleg was burned similarly, with the results shown in the solid lines, 1 and 1. At arrow C, the left hind leg was burned in order to see whether a simple extension of the lesion in an area remote from those in which lymph was being collected would indirectly influence lymph formation. This final burn had no recognizable effect, nor have we ever been successful in enhancing lymph flow from an

unburned region by even extremely severe burns in distant regions.

The normal mean arterial pressure in the carotid artery of the calf, anesthetized with nembutal, varies between 160 and 180 mm. Hg. In the experiment shown in Figure 3, it was 162 mm. Hg prior to burning, 182 mm. Hg 1 hour after the first burn (arrow A), 204 mm. Hg 5 hours after the first burn, and 156 mm. Hg 14 hours after the first burn and near the end of the experiment. No attempts were made to measure venous pressures before and after burning, but Field, Drinker, and White (11), in severe burns of the dog, found that venous pressures taken from large vessels immediately above the level of the burn rose rapidly following burning.

##### 5. Chemical changes

Complete data from two experiments are given in Tables II and III. Table IV gives a summary of the maximal changes observed in each experiment.

TABLE II  
Calf 2

Time	Volume		Protein refractometer		N.P.N.		Creatine* + creatinine		Creatinine		Urea N		Amino N		Amino N tungstate filtrate		
	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	
Before burn	4.5†	7.3†	2.2†	2.8†	16.6		3.5		1.2		8.7		31.8		7.4		
Lymph	Left foreleg burned 4 minutes																
	minutes																
	0 to 15	12.6		3.78		20.8		12.5		1.2		8.9		44.6		6.9	6.8
	15 to 30	12.7	7.9		3.20	25.0	19.8	13.2	4.4	1.3	0.7	9.4	9.4	43.9	38.7	7.2	
	30 to 45	11.5		3.62		24.8		12.8									
	45 to 60	11.5	5.0		2.89	24.3	20.4	12.9	4.4								
	hours																
	1 to 2	41.0	8.0	3.70	3.04	26.2	23.8	12.9	4.7			10.0	9.9		38.0	7.6	7.4
	2 to 3	36.0	7.0	3.72	2.84	27.2	28.2	11.1	4.2	1.1	0.7	11.7		45.2		8.7	8.9
	3 to 3½	18.4	3.3	3.70	2.74	26.6	26.9	10.9	4.9								
	Right foreleg burned 4 minutes																
	4 to 5	30.0	43.0	3.68	4.22	29.6	34.8	12.6	10.5			12.8				8.5	10.3
	5 to 7	50.0	77.0	3.90	4.30												
	7 to 10	65.0	94.0	3.90	4.30	41.2	47.1	13.5	13.6								
	10 to 14	76.0		3.80													
	14 to 18	53.0	82.0	3.74	3.97	58.0	61.4	15.7	15.0	4.8		35.8	38.1	45.6	47.3	10.4	11.2
Serum	0		6.04		17.7		3.1		1.3		8.3		57.5		7.9		
	7				38.3		9.3		2.6		21.1		57.1		7.9		
	18		5.34		62.2		17.0		4.8		41.0		56.3		7.5		

\* Creatine + creatinine given as creatinine.

† Average per hour.

The non-protein nitrogen increased in all experiments. This increase occurred at about the same rate in lymph from both the burned and unburned leg and in the serum. The values for "end serum" are usually somewhat higher than those for the last lymph samples. This is to be expected when there is a continuous rise in non-protein nitrogen, since the last lymph sample contains all the lymph taken during the last 2 to 4 hours while the "end serum" is taken at the time the experiment was terminated.

The case of creatine and creatinine is not quite as clear. In most cases, a slow increase which occurs at about the same rate in both legs is observed. In the case of calf 2, as shown in Table II, there is an immediate increase in creatine from the burned leg, and this level is maintained with only a small further increase toward the end of the experiment. Calf 3, which survived for only 4 hours, showed a similar picture.

The results of the amino-N determinations are irregular. In some cases, the amount in the lymph from the burned leg was higher than in the unburned; in other cases, the opposite was true.

These differences were not large enough to be significant. Surprising, however, is the difference between the concentration in lymph and serum at the end of the experiments (Table IV). Calves 2, 5, 6, and 7 have a higher concentration of amino-N in the lymph than in the serum. The lymph samples for calves 4 and 9 were taken some hours before the serum sample, leaving only calf 3 with a much higher concentration of the amino-N in the serum than in the lymph. As already mentioned, calf 3 died after only 4 hours; it should also be pointed out in this connection that there was considerable hemoglobinemia, there being a concentration of 2.3 per cent hemoglobin in the serum.

A control experiment was carried out to determine how much effect the nembutal anesthesia in itself would have on the different blood and lymph constituents. Very small changes only were observed. None of the changes encountered in the burned animals can be explained as a result of the anesthesia.

Our original assumption was that compounds set free by tissue damage in the burned area would

be present in the lymph from this area in higher concentration than in the lymph from an unburned area or in the serum from the general circulation. If this is correct, then our results indicate that the increase in the compounds studied, with the possible exception of creatine, is due to metabolic changes elsewhere in the body.

It is generally assumed that urea is formed in the liver and not in other tissues and there would, therefore, be no reason to expect any formation of urea in the burned area. The increase in urea and creatinine could both be explained as an accompaniment of the oliguria which regularly occurs after severe burns. The very high nitrogen output reported for patients with burns (12) indicates increased protein catabolism, and the increase in urea might be due to a combination of increased formation and decreased excretion.

The relative increase in creatinine is about the

same as the relative increase in total non-protein nitrogen. On the other hand, the increase in creatine is both relatively and absolutely greater than the increase in creatinine. It does not seem likely that this can be explained as a result of oliguria. Creatine is not normally excreted in the urine, but Chanutin and Silvette (13) found, in spite of this, that nephrectomy caused an increase in the creatine as well as in the creatinine of the blood, though the increase in creatine was less than that in creatinine. This result resembles the usual findings in nephritis and Miller and Dubos (14) have shown that in cases of nephritis as much as 30 per cent of the color produced in the Jaffe reaction may be due to compounds other than creatinine. We performed a few tests using Miller and Dubos's specific bacterial enzyme to prove the identity of the creatine and creatinine. The "end serum" from calf 9 was used. All the

TABLE III  
Calf 9

Time	Volume		Protein refractometer		N.P.N.		Creatine* + creatinine		Creatinine		Urea N		Amino N tungstate filtrate		
	L	R	L	R	L	R	L	R	L	R	L	R	L	R	
Before burn	cc.		per cent		mgm. per cent										
	17.3†	18.0†	2.99†	2.98†	13.7		3.9		0.9		4.8		5.1		
Lymph	Right foreleg burned 3½ min.														
	0 to 1	17.0	23.0	2.73	3.94	15.7	16.1	4.5	5.0	1.0	1.0	5.6	5.5	5.7	5.7
	Right hind leg burned 3½ min.														
	1 to 2	19.0	33.0	2.71	3.66			5.8	6.3			5.5	5.4	7.1	7.1
	2 to 3	11.0	27.0	2.71	3.92										
	3 to 4	13.0	27.0	2.62	3.72										
	4 to 5	8.5	28.0	2.57	3.81										
	5 to 6	11.0	29.0	2.54	3.81	24.2	23.7	7.6	6.3			9.4	8.7	7.9	8.4
	6 to 7	8.0	28.5	2.57	3.74										
	7 to 8	8.0	23.5	2.53	3.65									7.3	7.1
	8 to 9	10.0	25.0	2.54	3.74										
	9 to 10	7.5	20.0	2.54	3.76										7.0
	10 to 12	15.0	38.0	2.69	3.76										
	12 to 14	10.0	34.0	2.82	3.70										
	14 to 16	16.0	30.0	2.77	3.48										
	16 to 18	17.0	30.0	2.66	3.48	39.3	38.0	10.3	9.2	2.5	2.2	24.2	20.9	7.4	9.2
	18 to 20	20.0	34.0	2.89	3.44										
20 to 22	16.5		2.76												
23 to 26	18.0	43.0	2.78	3.50											
26 to 29	15.0	29.0	2.62	3.50	55.8		17.0					38.6	8.5	9.0	
29 to 32	18.0	25.0	2.59	3.44	65.5		21.0				44.3		8.4		
32 to 35	13.0	17.0	2.74	3.48									11.8	11.4	
35 to 38															
Serum	0		6.34		14.3		3.8		0.9		4.9		4.9		
	9½		6.20		25.5		6.1		1.4		13.6		6.6		
	23½		6.25								33.9		8.4		
	38		6.22		90.1		33.0		6.2		55.7		12.7		

\* Creatine + creatinine given as creatinine.

† Average per hour.

TABLE IV

## Summary of analytical data

Normal serum and lymph were taken before burn, end serum was taken at the termination of the experiment, and end lymph was collected from the burned leg during a 3 to 4-hour period just prior to the termination of the experiment except in the cases where actual time is given in footnote

Calf number	Time after initial burn	Protein		N.P.N.		Creatinine		Creatine + creatinine		Urea N		Amino N in tungstate filtrate		Remarks
		Normal	End	Normal	End	Normal	End	Normal	End	Normal	End	Normal	End	
1	15½	<i>per cent</i>						<i>mgm. per cent</i>						Burned left foreleg 2½ minutes; 3 hours later, right foreleg 2 minutes
		Lymph 2.42	3.91	16.7	73.5	1.0	4.5	3.2	22.5					
		Serum 5.39	6.04	16.7	84.8	1.1	4.9	2.9	25.5					
2	18	Lymph 2.20	3.74	16.6	58.0	1.2	4.8	3.5	15.7	8.7	36.8	7.4	10.4	Burned left foreleg 4 minutes; 4 hours later, right foreleg 4 minutes
		Serum 6.04	5.34	17.7	62.2	1.3	4.8	3.1	17.0	8.3	41.0	7.9	7.5	
3	4	Lymph 2.34	3.97	27.6	54.0	1.5	2.6	4.5	20.2	16.8	22.7	5.1	12.4	Burned left hind leg 3 minutes; right hind leg 3 minutes; 1 hour later, right foreleg 3 minutes
		Serum 6.10		26.0	67.8	1.3	3.9	4.1	21.6	17.2	26.0	3.7	20.4	
4	11	Lymph 3.34	4.36	27.5	72.3*	1.0	3.8	4.5	21.5	13.9	29.7†	6.3	16.5†	Burned left foreleg 3 minutes; 1½ hours later, left hind leg 3 minutes; 8 hours later, right hind leg 3 minutes
		Serum 6.75	6.43	28.6	96.8	1.0	5.7	4.5	27.5	13.5	45.3	6.0	18.5	
5	10	Lymph 2.42	3.88	19.7	38.6	1.1	2.1	4.3	9.8			5.6	11.9	Burned right foreleg 3 minutes; 5 hours later, left hind leg 1½ minutes; 2 hours later, right hind leg 1½ minutes
		Serum 6.38	5.34	20.5	41.8	1.3	2.9	3.9	12.0	11.3	19.8	5.3	9.0	
6	17	Lymph 2.46	3.56	18.9	41.7	1.2	2.4	4.5	9.9	9.4	24.4	6.0	12.3	Burned right foreleg 3½ minutes; 12 hours later, right hind leg 3½ minutes
		Serum 6.30	4.76	19.8	48.4	0.9	2.6	3.9	12.3			5.8	8.9	
7	15½	Lymph 2.96	3.80	21.4	37.5	0.9	1.8	4.8	8.3			7.3	9.7	Burned left foreleg 3 minutes; 6 hours later, right foreleg 3 minutes; 2½ hours later, left hind leg 3 minutes
		Serum 7.04	6.24	19.9	36.3			5.1	8.4			5.4	6.8	
9	38	Lymph 2.98	3.48	13.7	65.5‡	0.9		3.9	21.0‡	4.8	44.3‡	5.1	8.4‡	Burned right foreleg 3½ minutes; 1 hour later, right hind leg 3½ minutes
		Serum 6.34	6.22	14.3	90.1	0.9	6.2	3.8	33.0	4.9	55.7	4.9	12.7	

\* Sample 6½ to 8½ hours.

† Sample 4½ to 6½ hours.

‡ Sample 29 to 32 hours.

chromogenic material, also that produced by acid hydrolysis, disappeared after incubation with the bacteria.<sup>2</sup> This would prove quite conclusively

<sup>2</sup> We wish to thank Dr. René Dubos for kindly presenting us with cultures of *Corynebacterium creatinovorans*.

that the chromogenic material was true creatine and creatinine.

The results from calf 2 show that it is possible, at least under certain conditions, to demonstrate that creatine is released in the burned area. Muscle cells are known to have a high concentration

of creatine and an injury to such cells, to the extent of releasing material usually confined to the inside of the cells, would result in an increase of creatine in the extracellular fluids. A possible explanation for our failure to observe this phenomenon in the majority of our experiments is that the creatine (and possibly other constituents) is diffused so rapidly from the tissue fluid into the capillaries that the differences in concentrations between blood and lymph are too small to be determined.

An increase of amino acid in the blood has been found in late stages of nephritis, but this is by no means a constant finding. Kirk (15), from his observations, concludes that the occurrence of amino acid in the blood of patients with nephritis is not due directly to renal failure but to a metabolic failure elsewhere. After nephrectomy, there is very little, if any, increase in amino acid (16, 17). Our experiments give no clue to the source of the amino nitrogen, but an increase in amino acid might result either from liver damage or from increased protein catabolism.

An increase in "undetermined nitrogen" out of proportion to the increase in total non-protein nitrogen has been found in cases of burns (18). In our experiments, the "undetermined nitrogen" does not increase proportionately more than the total non-protein nitrogen except in the case of calf 3.

#### CONCLUSIONS

Our studies indicate that there is no increase in capillary permeability in normal regions of the body distant from the burn. The increase in urea and creatinine is consistent with the usual findings attending oliguria. An explanation of the increase in creatine and amino nitrogen requires further studies which we are now undertaking. It is tentatively suggested that the increase in creatine is due to tissue damage in the burned area.

#### SUMMARY

1. A method of anesthesia and cannulation of the principal lymphatic draining the foreleg of the calf is described.
2. A method for providing steady passive motion of the legs and so inducing constant conditions of lymph flow is described and illustrated.

3. The technique of producing repeatable and reasonably quantitative burns of the leg of a calf weighing 125 to 200 pounds is described.

4. By means of a table and curves, the lymph flow and lymph protein content from normal and burned forelegs of calves are presented.

5. Non-protein nitrogen, urea, creatinine, creatine + creatinine, and amino nitrogen were determined in lymph from a burned area, in lymph from an area remote from the burn, and in serum. The results are presented in tabular form.

The authors wish to express their appreciation to Helen H. Gilbert and H. S. Amory Potter for technical assistance during experiments lasting through one and even two nights, and to Marion Blanchard and Esther Hardenbergh for aiding in chemical analyses.

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# IMPROVEMENTS IN CALCULATION OF RENAL RESISTANCE TO BLOOD FLOW. CHARTS FOR OSMOTIC PRESSURE AND VISCOSITY OF BLOOD

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Previously, we have offered formulas to determine the resistance to blood flow of the afferent and efferent arterioles of the kidney (1). They employed a modified Poiseuille's Law and were based especially on the concept of osmotic equilibrium in the glomerulus between the blood leaving the glomerular capillaries and the ultrafiltrate in Bowman's capsule. They utilized the inulin clearance as a measure of the rate of glomerular filtration and the diodrast clearance as a measure of the rate of renal plasma flow, as developed by Homer Smith and his coworkers (2, 3). For determining the osmotic pressure of blood both before and after glomerular filtration, we depended on the data of the Adairs and Greaves (4) for human serum diluted below normal, not having been able, as yet, to supplant them with observations on concentrated plasma.

The formulas for renal afferent and efferent arteriolar resistance were, respectively (1):

$$R_A = \frac{P_M - P_o - 40}{HD};$$

$$R_E = \frac{(1 - 0.47F)(P_o - P_o + 10)}{HD}.$$

Here  $P_M$  is the mean of systolic and diastolic blood pressure in mm. Hg,  $D$  is the effective renal plasma flow (diodrast clearance, usually), and  $H$  is the reciprocal of  $(1 - \text{hematocrit})$ .  $F$  is the glomerular filtration fraction (the ratio of inulin to diodrast clearance),  $P_o$  is the osmotic pressure in mm. of Hg of the blood after concentration by glomerular filtration, and  $P_o$  is the value before glomerular concentration. The osmotic pressures were given in terms of a formula which depended on the serum protein and filtration fraction, the use of which will be discussed later. The unit for  $R_A$  and  $R_E$  depends on the unit for  $D$ . Ordinarily, it will be mm. Hg per

cc. per minute per 1.74 square meters of body surface.

## Poiseuille's Law

In our original use of Poiseuille's law, we followed Whittaker and Winton's (5) description of the viscosity of blood of varying hematocrit (1) in the pump-lung-limb of the dog. They found that pressure and flow were strictly proportional for Ringer's solution, but that a constant had to be subtracted from the pressure when whole blood was used. This constant was about 20 mm. Hg for normal hematocrits but of course it decreased with the hematocrit so that for nearly zero hematocrit (plasma), it was not far from zero. At the time of the formulation of our equations for resistance, we were not aware of the similarity between Whittaker and Winton's *in vivo* findings for blood and the large body of observation in the field of physical chemistry concerning the flow of mixtures, emulsions, suspensions, and plastic solids. These facts are summarized in a monograph by Bingham (6) on which we draw. Similar conclusions are found elsewhere (7).

The flow-pressure curve of a plastic solid is identical in form with the almost linear curves for blood found by Whittaker and Winton. Bingham gives the relationship (p. 225, with modification) as:

$$\text{Resistance} = \frac{\text{Perfusion pressure} - \text{Yield pressure}}{\text{Rate of flow} \times \text{Viscosity}}.$$

Apparently, part of the applied pressure is used in producing viscous flow and part in overcoming the internal friction of the red cells. As a whole, there is plastic flow. This equation would imply that no flow would occur at low values of pressure, but this is not actually true in plastic solids and Bingham offers various explanations for the observed continuance of slight flow at low pressures. (One of them is the same

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as Whittaker and Winton's suggestion in their discussion of the low apparent viscosity of blood *in vivo*.) Furthermore, this formula is not quite correct slightly above the yield pressure. It applies only to the linear portion of the pressure-flow curve.

As has been indicated, we have offered no formula to express yield pressure in terms of the hematocrit, having had no basis on which to do so. Our formula used a constant quantity, 20 mm. Hg, obtained from Whittaker and Winton's findings for normal hematocrits, as the value for yield pressure. Bingham's analysis, however, enables us to make progress by using a variable rather than a static yield pressure.

He defines  $f$  as the internal friction in the plastic in terms of shear. It is a constant, independent of the viscometer capillary's dimensions and is related to  $P_f$ , the yield pressure, as follows, where  $r$  is the radius of the bore of the capillary viscometer and  $L$  is its length:

$$f = \frac{r}{2L} P_f.$$

Also, where  $A$  and  $B$  are constants, independent of the viscometer and  $H_c$  is the hematocrit,

$$f = A + BH_c,$$

since  $f$  is linearly related to the volume concentration of the suspended substance (6). Then,

$$P_f = \frac{2L}{r} (A + BH_c).$$

Since  $P_f$  is nearly zero when  $H_c$  is zero (if it is not actually zero—back-pressure due to edema and tissue elasticity may cause an artifact), we can set  $A = 0$ . Then,

$$P_f = \frac{2L}{r} BH_c.$$

In this application, we have imagined the portion of the blood vascular system we are considering to be replaced by an ideal viscometer of uniform bore (radius  $r$ ), length  $L$ , and resistance to blood flow  $T$ . If we always use the same bore of our ideal equivalent viscometer,  $r$  is the same for the same degree of vasoconstriction, and vary its length to adjust for differences in resistance in organs and regions, the resistance being studied,  $r$  will represent the dog

dilatation and  $1/r$  the degree of vasoconstriction. Such a model indicates that the yield pressure in the study of a particular organ is proportional to the hematocrit and to the degree of vasoconstriction.

Now, Bingham shows that for plastic solids, as for viscous fluids, the resistance is proportional to the length and inversely proportional to the fourth power of the radius of the capillary viscometer. Thus:

$$T = L/b^4 r^4.$$

Here  $b$  is a constant. We then have:

$$P_f = bBH_c(T/L)^{1/4}.$$

The temptation, at this point, is to evaluate the unknown quantity,  $bB/L^{1/4}$ , by using Whittaker and Winton's data; set  $P_f = 20$  mm. Hg,  $H_c = \frac{1}{2}$ ; calculate  $T$  (the resistance of the hind-limb of the dog) and insert it in the above formula. But such a method, while entirely legitimate for the hind-limb preparation, is not applicable to the kidney, since both  $L$  and  $T$  are dependent on the region in which they are measured. What is true of the hind-limb does not apply to the kidney, so far as  $L$  and  $T$  are concerned. Actually, the replacement of  $r$  by  $T$ , *et cetera*, in the expression for  $P_f$  is not really an improvement unless the expression can be evaluated by means of a kidney pressure-flow curve, similar to Whittaker and Winton's for the hind-limb of the dog, the intercept of which would give us the yield pressure for a given degree of vasoconstriction (flow = 0; pressure =  $P_f$ ). The possibility of obtaining such pressure-flow curves for the kidney seems remote. That organ, the vessels of which appear to react autonomously to changes in blood pressure, would not maintain constancy of vessel size (constant resistance) while the pressure was being varied. Clearly, an approximation to yield pressure in the kidney must be used, if it is justifiable.

Our formula for  $P_f$ , above, shows that, for a given  $L$  (constant),  $P_f$  varies as the fourth root of the resistance to blood flow (when  $L$  is constant). That is, if the resistance to blood flow (when  $L$  is constant) is increased 50 per cent,  $P_f$  increases 11 per cent. If  $P_f$  is 20 mm. Hg, the increase in the

leg preparation with normal hematocrit, a 50 per cent increase in resistance would augment  $P_f$  by about 2 mm. Hg. Such a quantity seems unimportant at the present stage of biological precision in renal physiology. The greatly increased difficulty in computation resulting from modifying the yield pressure by the fourth root of the resistance would hardly compensate for the slight improvement except in highly abnormal circumstances.<sup>2</sup> We therefore can neglect for ordinary physiological purposes the variable effect on yield pressure of the resistance, retaining solely the hematocrit.

We continue to use 20 mm. Hg for the yield pressure, the figure obtained from Whittaker and Winton when the hematocrit is normal (0.43). Although this may not be the best value for the kidney, it is the best available and is probably not far wrong, since we have seen that the yield pressure is relatively insensitive to changes in resistance. While the kidney has a high rate of blood flow for its size, compared to muscle (the hind limb of the dog), Whittaker and Winton's preparations were studied in a state of dilatation so that comparison between the two sites may be justified as an approximation within our present limits of precision.<sup>3</sup> We therefore write for  $P_f$  the following expression (based on  $P_f = 20$ , when  $H_c = 0.43$ ):

$$P_f = 46H_c.$$

Since the blood pressure is reduced to the usual capillary pressure, in passing through the kidney, in two stages—the afferent and the efferent arterioles—rather than one, it is advisable to allocate  $P_f$  between the afferent and efferent arteriolar resistances, neglecting the small capillary and venous terminal portion ( $R_T$ ). Our previous formulas for  $R_A$  and  $R_E$ , the afferent and efferent arteriolar resistances, were obtained by allotting all of the fixed yield pressure (20 mm.

Hg) to the afferent arteriolar resistance.<sup>4</sup> Under this circumstance,  $R_A$  is slightly larger than  $R_E$  (9). If  $P_f$  is distributed between  $R_A$  and  $R_E$ , as now seems advisable, this preponderance of  $R_A$  over  $R_E$  is enhanced. Whether the afferent arterioles are of greater length or greater constriction (or both) than the efferent arterioles is not clear, but in view of the few millimeters of mercury difference in yield pressure between one possibility and the other, at this time, for lack of better information, an equal partition of  $P_f$  between  $R_A$  and  $R_E$  seems wise.

Accordingly, we have:

$$R_A = \frac{\text{Perfusion pressure afferent arterioles} - \frac{1}{2}P_f}{\text{Rate of flow} \times \text{Viscosity}};$$

$$R_E = \frac{\text{Perfusion pressure efferent arterioles} - \frac{1}{2}P_f}{\text{Rate of flow} \times \text{Viscosity}}.$$

Since  $\frac{1}{2}P_f = 23H_c$ , the formulas become:

$$R_A = \frac{P_M - P_o - 23H_c - 20}{HD};$$

$$R_E = \frac{(1 - 0.47F)(P_o - P_o - 23H_c + 10)}{HD}.$$

While these formulas supersede the previous ones, it is unlikely that they would seriously modify the results obtained from the older version, being a refinement based on a more precise value for yield pressure and its allocation to both afferent and efferent arteriolar resistances.

#### *Post-arteriolar renal resistance to blood flow*

The formulas developed have been limited to arterial and arteriolar resistance; they did not include the resistance of the kidney to blood flow after the glomerular filtrate had been reabsorbed in the capillaries and the blood flowed on into the renal vein, outside the kidney. But the same methods can be applied to evaluate this post-arteriolar resistance.

It will be, as before, the perfusion pressure less the yield pressure, divided by the rate of blood flow times viscosity. Or, where  $R_T$  is this last portion of renal resistance referred to the sub-

<sup>2</sup> Should evidence appear indicating the value of such a modification, a fairly good approximation may be made by using

$$P_f = \text{Constant} \times H_c \frac{(\text{Perfusion pressure} - \text{Constant} \times H_c)^{\frac{1}{4}}}{\text{Flow}}.$$

<sup>3</sup> The relatively low resistance of the kidney to blood flow also suggests that the linear portion of the pressure-flow curve is normally present.

<sup>4</sup> The effect of glomerular capillary resistance, which has been included in the expression for afferent arteriolar resistance, has been assayed in the published report (1), though it was probably not available to Shannon, who no doubt depended on an earlier manuscript version, when noting its absence in a recent review of renal physiology (8).

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Also, where  $A$  and  $B$  are constants, independent of the viscometer and  $H_c$  is the hematocrit,

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In this application, we have imagined the portion of the blood vascular system we are considering to be replaced by an ideal viscometer of uniform bore (radius  $r$ ), length  $L$ , and resistance to blood flow  $T$ . If we always choose the bore of our ideal equivalent viscometer so that it is the same for the same degree of vasoconstriction, and vary its length to adjust for the different organs and regions, the resistance of which is being studied,  $r$  will represent the degree of vaso-

dilatation and  $1/r$  the degree of vasoconstriction. Such a model indicates that the yield pressure in the study of a particular organ is proportional to the hematocrit and to the degree of vasoconstriction.

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$$T = L/b^4r^4.$$

Here  $b$  is a constant. We then have:

$$P_f = bBH_c(T/L)^{\frac{1}{4}}.$$

The temptation, at this point, is to evaluate the unknown quantity,  $bB/L^{\frac{1}{4}}$ , by using Whittaker and Winton's data; set  $P_f = 20$  mm. Hg,  $H_c = \frac{1}{2}$ ; calculate  $T$  (the resistance of the hind-limb of the dog) and insert it in the above formula. But such a method, while entirely legitimate for the hind-limb preparation, is not applicable to the kidney, since both  $L$  and  $T$  are dependent on the region in which they are measured. What is true of the hind-limb does not apply to the kidney, so far as  $L$  and  $T$  are concerned. Actually, the replacement of  $r$  by  $T$ , *et cetera*, in the expression for  $P_f$  is not really an improvement unless the expression can be evaluated by means of a kidney pressure-flow curve, similar to Whittaker and Winton's for the hind-limb of the dog, the intercept of which would give us the yield pressure for a given degree of vasoconstriction (flow = 0; pressure =  $P_f$ ). The possibility of obtaining such pressure-flow curves for the kidney seems remote. That organ, the vessels of which appear to react autonomously to changes in blood pressure, would not maintain constancy of vessel size (constant resistance) while the pressure was being varied. Clearly, an approximation to yield pressure in the kidney must be used, if it is justifiable.

Our last expression for  $P_f$ , above, shows that, for a given site ( $L$  constant),  $P_f$  varies as the fourth root of the resistance to blood flow (when on the linear portion of the pressure-flow curve). That is to say,  $P_f$  is not very sensitive to changes in resistance. Thus, a 50 per cent increase in resistance entails an increase in  $P_f$  of 11 per cent. If  $P_f$  is 20 mm. Hg, its approximate value in the

leg preparation with normal hematocrit, a 50 per cent increase in resistance would augment  $P_f$  by about 2 mm. Hg. Such a quantity seems unimportant at the present stage of biological precision in renal physiology. The greatly increased difficulty in computation resulting from modifying the yield pressure by the fourth root of the resistance would hardly compensate for the slight improvement except in highly abnormal circumstances.<sup>2</sup> We therefore can neglect for ordinary physiological purposes the variable effect on yield pressure of the resistance, retaining solely the hematocrit.

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Accordingly, we have:

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$$R_E = \frac{\text{Perfusion pressure efferent arterioles} - \frac{1}{2}P_f}{\text{Rate of flow} \times \text{Viscosity}}.$$

Since  $\frac{1}{2}P_f = 23H_c$ , the formulas become:

$$R_A = \frac{P_M - P_o - 23H_c - 20}{HD};$$

$$R_E = \frac{(1 - 0.47F)(P_o - P_o - 23H_c + 10)}{HD}.$$

While these formulas supersede the previous ones, it is unlikely that they would seriously modify the results obtained from the older version, being a refinement based on a more precise value for yield pressure and its allocation to both afferent and efferent arteriolar resistances.

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It will be, as before, the perfusion pressure less the yield pressure, divided by the rate of blood flow times viscosity. Or, where  $R_v$  is this last portion of renal resistance referred to the sub-

<sup>2</sup> Should evidence appear indicating the value of such a modification, a fairly good approximation may be made by using

$$P_f = \text{Constant} \times H_c \frac{(\text{Perfusion pressure} - \text{Constant} \times H_c)^{\frac{1}{4}}}{\text{Flow}}.$$

<sup>3</sup> The relatively low resistance of the kidney to blood flow also suggests that the linear portion of the pressure-flow curve is normally present.

<sup>4</sup> The effect of glomerular capillary resistance, which has been included in the expression for afferent arteriolar resistance, has been assayed in the published report (1), though it was probably not available to Shannon, who no doubt depended on an earlier manuscript version, when noting its absence in a recent review of renal physiology (8).

ject's own blood as perfusion standard (viscosity = 1), and  $P_V$  is the pressure of the renal vein in mm. of Hg, we have:

$$R_V = \frac{(\text{Initial pressure} - \text{Terminal pressure}) - \text{Yield pressure}}{HD}$$

Because of the relatively slight resistance after the arterioles, it seems inadvisable to allocate any of the previously discussed yield pressure to  $R_V$ , so that this term drops out.

$$R_V = \frac{\text{Initial pressure} - P_V}{HD}$$

This initial pressure is clearly the same as the terminal pressure for efferent arteriolar resistance, which was  $P_o + P_R$ , that is—the osmotic pressure of the systemic blood plus the renal interstitial pressure, considered to be 20 mm. Hg. We therefore have:

$$R_V = \frac{P_o - P_V + 20}{HD}$$

Such a formula is, of course, an approximation. It suggests, since the numerator is constant, that the capillaries and venules dilate with increased blood flow so as to reduce the resistance in proportion to the increased flow. Actually, this is not completely true. Our definition of efferent arteriolar resistance, by using a fixed terminal pressure, is more functional than anatomical in scope. With a marked increase in blood flow, for example, it is likely that this fixed pressure point shifts along the capillaries, towards the venous end, so that anatomically a portion of what was originally included in  $R_V$  would be shifted to  $R_E$ . So long as the limitations of our definitions of  $R_E$  and  $R_V$  are explicit and remembered no harm can result.

Total renal resistance now can be defined:

$$R_K = R_A + R_E + R_V.$$

In this way, total resistance from the renal artery to the renal vein has been evaluated.

#### *Osmotic pressure affected by A : G ratio*

The formulas for renal resistance depend on an expression for the osmotic pressure of blood, derived from one offered by the Adairs and Greaves (4) from measurements on serum of a

particular albumin-globulin ratio. We made no correction for A : G ratios differing from theirs of 2.20. While the discrepancy thereby resulting is usually not large, we have been able to minimize it by applying an empirical formula of Wies and Peters (10) for the osmotic pressure of a large range of A : G ratios in human sera. They found that albumin was 2.66 times as effective as globulin in elevating osmotic pressure in the range studied.

Let us call the concentration of serum proteins  $\bar{S}$  in grams per 100 cc. with an A : G ratio of  $B$ . Let  $S$  be the concentration of serum protein, in grams per 100 cc., which would be required of the Adairs' and Greaves' 2.20 A : G ratio serum (our standard) to have the same osmotic pressure as the sample with the  $B$  ratio. Let  $g$  be the number of millimeters of mercury osmotic pressure contributed per gram globulin in serum.

We then have:

1 gram globulin produces  $g$  mm. Hg osmotic pressure.

1 gram albumin produces 2.66  $g$  mm. Hg osmotic pressure.

1 gram protein of 2.2 A : G ratio contains 2.2/3.2 grams albumin, and 1/3.2 grams globulin.

1 gram protein of  $B$  A : G ratio contains  $\frac{B}{B+1}$  grams albumin and  $\frac{1}{B+1}$  grams globulin.

1 gram protein of 2.2 A : G ratio has an osmotic pressure of

$$\frac{2.2}{3.2} \times 2.66g + \frac{1}{3.2} \times g.$$

1 gram protein of  $B$  A : G ratio has an osmotic pressure of

$$\frac{B}{B+1} \times 2.66g + \frac{1}{B+1} \times g.$$

It is therefore clear that 1 gram of protein of A : G ratio  $B$  can be replaced in computing its osmotic pressure by

$$\left( \frac{B}{B+1} \times 2.66g + \frac{1}{B+1} \times g \right) \div \left( \frac{2.2}{3.2} \times 2.66g + \frac{1}{3.2} \times g \right)$$

grams of protein of 2.20 A : G ratio. That is to

RENAL RESISTANCE. OSMOTIC PRESSURE AND VISCOSITY OF BLOOD

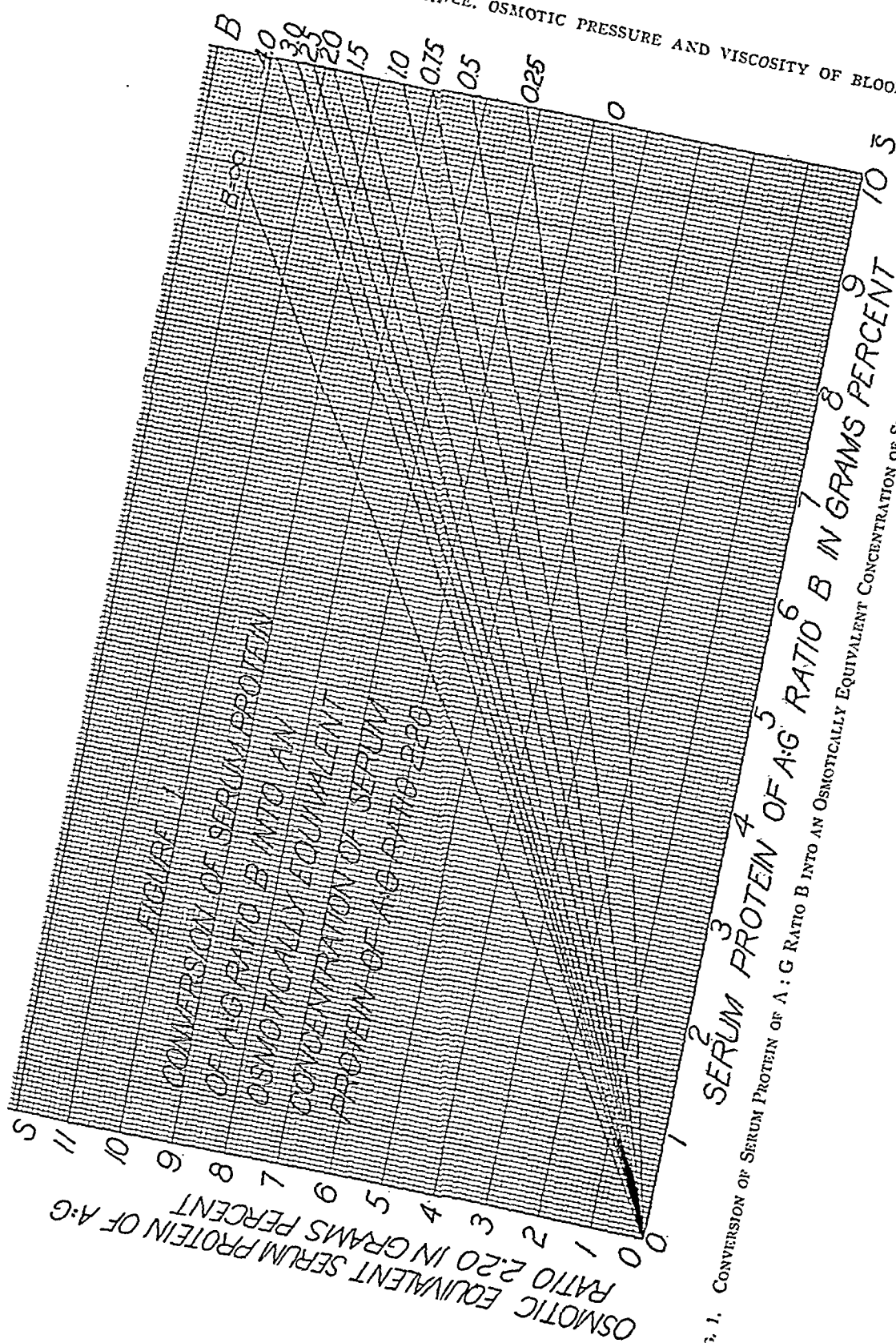


FIG. 1. CONVERSION OF SERUM PROTEIN OF A:G RATIO 2.20 INTO AN OSMOTICALLY EQUIVALENT CONCENTRATION OF SERUM PROTEIN OF A:G RATIO 2.20



say:

$$S = \frac{1 + 2.66B}{1 + B} \times \frac{3.2}{1 + 2.2 \times 2.66} \bar{S}.$$

Or,

$$S = \frac{0.467 + 1.243B}{1 + B} \bar{S}.$$

This conversion formula is represented graphically in Figure 1, whereby we obtain the osmotic equivalent in terms of serum protein of A : G ratio 2.20 for sera of varying A : G ratio *B*.

The osmotic pressure in mm. of Hg of plasma, at blood temperature, where the serum protein has the A : G ratio of 2.20, our *S*, is (1):

$$P_o = \frac{2.34S}{1 - 0.0542S}.$$

In the glomerulus, a protein-free filtrate leaves the blood plasma, which is thereby concentrated. The fraction of plasma filtered off as glomerular filtrate (*F*) is measured by the ratio of inulin clearance (glomerular filtration rate) to diodrast clearance (rate of effective renal plasma flow). The osmotic pressure of the plasma leaving the glomerulus (*P<sub>o</sub>'*) is given by the formula:

$$P_o' = \frac{2.34S}{1 - 0.0542S - F}.$$

This formula, for constant values of *P<sub>o</sub>'*, is represented by straight lines relating *S* and *F* and is shown in Figure 2. It is clear that when *F* is set equal to zero, *P<sub>o</sub>'* becomes identical with

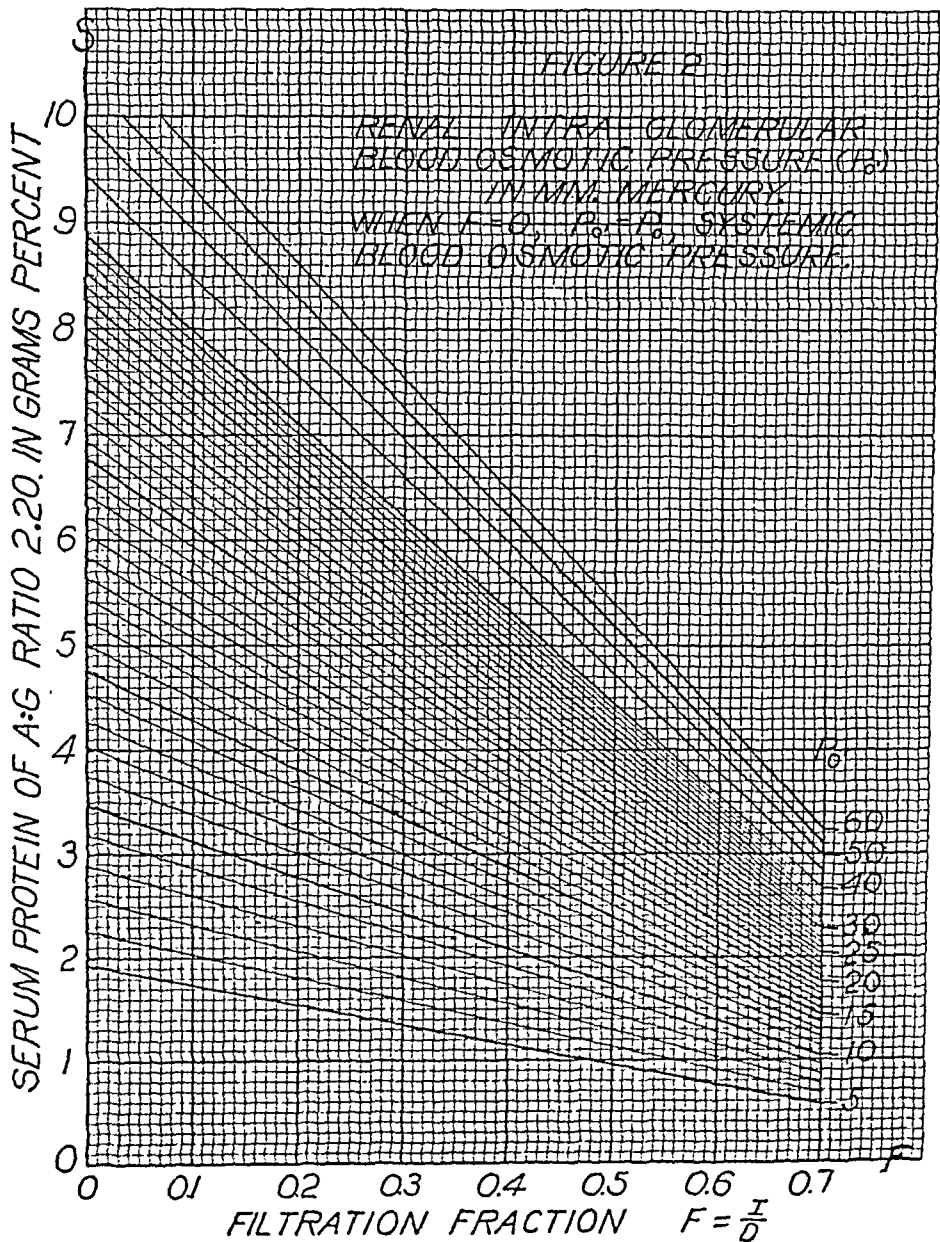


FIG. 2. RENAL INTRA-GLOMERULAR BLOOD OSMOTIC PRESSURE

$P_o$  (the osmotic pressure of the systemic blood). Thus, for a given  $S$  and  $F$ , Figure 2 can be used to determine the corresponding values of both  $P_o$  and  $P_{o'}$ . The value of  $S$ , of course, would have come from Figure 1 by the insertion of the observed quantities  $\bar{S}$  and  $B$ , the concentration of serum protein and its A : G ratio.

An actual example will illustrate the use of these charts. If the serum protein is 6.0 grams per 100 cc. with an A : G ratio of 1.5, from Figure 1 we find that the osmotically equivalent protein concentration with an A : G ratio of 2.20 is 5.6. This value for  $S$ , in Figure 2, is found to give a value for  $P_o$  (when  $F = 0$ ) of 18.9 and, if  $F = 0.2$ , for example, a value for  $P_{o'}$  of 26.4. These quantities would then be utilized in the resistance formulas.

#### VISCOSITY

The present use of the resistance formulas has involved either the comparison of the subject with himself as control, after a brief interval for the experimental procedure, or the assumption that little change in blood viscosity has occurred (1, 11). In certain instances, where the hematocrit and serum protein are different and still a comparison of renal resistance is desired, the formulas may not be applicable unless their strict meaning is remembered.

As derived,  $R_A$  and  $R_E$  refer to the resistance of the afferent and efferent arterioles to the flow of the subject's own blood taken as the viscosity standard. If his blood changes, the values of  $R_A$  and  $R_E$  will refer to the changed blood as standard. Such a definition has real physiological value, since our interest in measuring resistance is certainly in part prompted by the desire to understand how much flow any given pressure will produce of the subject's own blood, irrespective of its viscosity. Thus, if the blood viscosity were halved while  $R_A$  and  $R_E$  were found to be unchanged, we should be able to say that, for the same pressure, the same rate of blood flow through the arterioles would persist. Such a condition, however, could be attained only by *constriction* of the arterioles. In the hydraulic sense, referred to some arbitrary unit, the resistance of the arterioles in our example must certainly have increased, when the blood viscosity was halved, to prevent increase in renal blood

flow. We see, then, that the "hydraulic" resistance is necessary, if blood viscosity shifts, and we are interested in knowing the change produced in the calibre of the renal arterioles.

We are in a position to make this correction since we have a tentative expression for whole blood viscosity in terms of serum protein ( $S$ ) and the hematocrit ( $1/H = 1$ -hematocrit) (1). Let us call  $U$  the viscosity of whole blood and  $R_a$  and  $R_e$  the afferent and efferent "hydraulic" arteriolar resistances referred to a fixed viscosity perfusion standard as compared with our present "individual" formulations,  $R_A$  and  $R_E$ , which refer to the individual's own blood as viscosity standard. The small and large letter subscripts elsewhere will correspond analogously. We then have (1):

$$R_a = R_A/U; \quad R_e = R_E/U;$$

and where total "hydraulic" arteriolar resistance is  $R_r$ ,

$$R_r = R_a + R_e = R/U,$$

where  $R = R_A + R_E$ . And similarly for the post-arterial resistance:

$$R_v = R_V/U; \quad R_k = R_K/U.$$

Since the relative change in blood viscosity as a result of glomerular hemoconcentration is already included in the expression for  $R_E$ , we need for  $U$  only systemic blood viscosity, as previously offered (1).

$$U = \text{plasma viscosity} \times (0.27 + 0.983H). \\ U = (0.60 + 0.204S)(0.27 + 0.983H).$$

Or,

$$U = (1 + 0.34S)(0.162 + 0.590H).$$

While this formula can be used to change  $R_A$ , and the other quantities, into  $R_a$ ,  $R_e$ , and the corresponding analogues by computation, as shown above, the labor can be reduced by using Figure 3, where, for known values of serum protein and  $H$  (the hematocrit constant), the viscosity  $U$  is given. In this way, values for "hydraulic" resistance of the renal arterioles and the rest of renal resistance can be obtained, which give a notion of the calibre of these vessels, irrespective of the viscosity of the blood coursing through them.

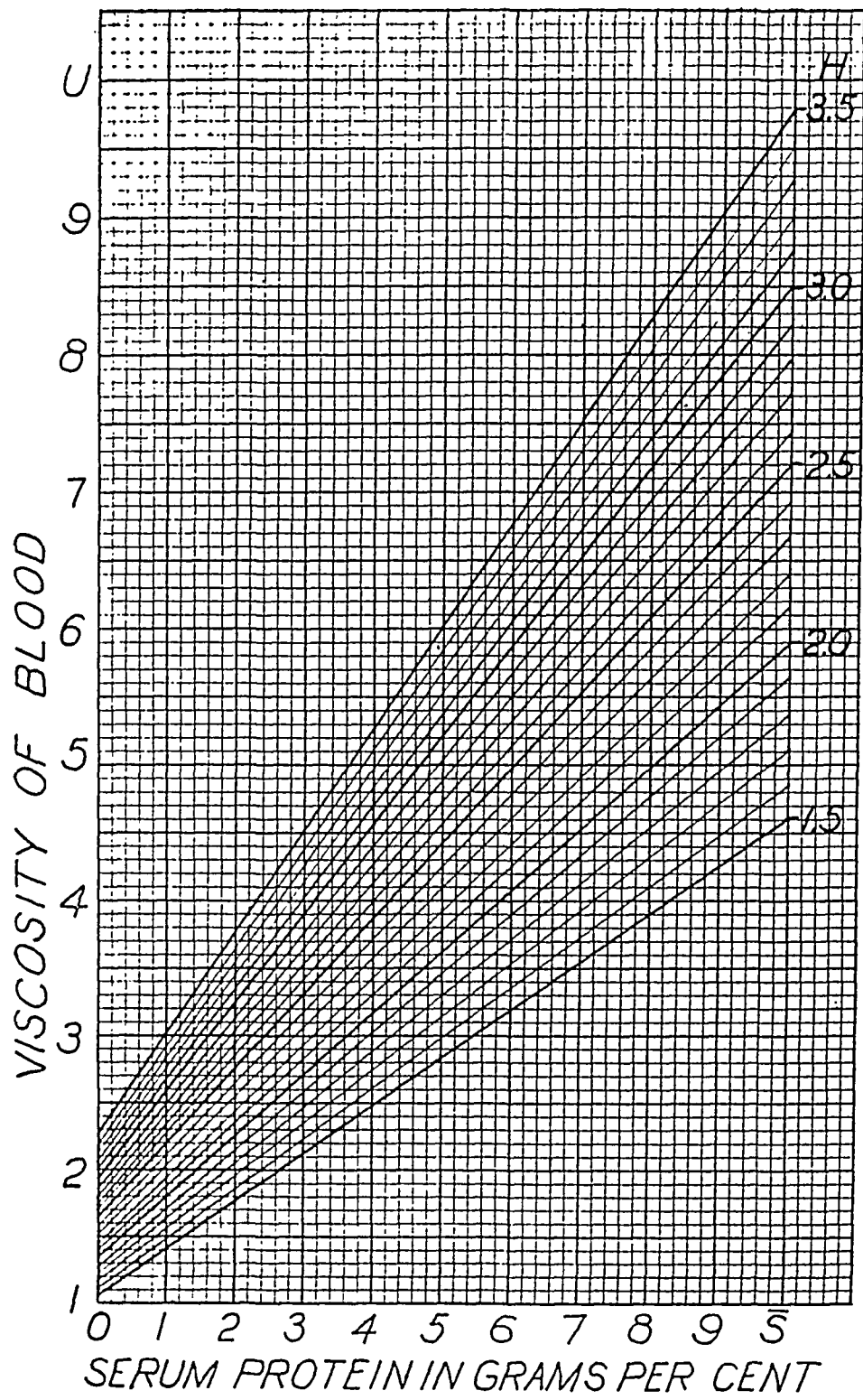


FIG. 3. VISCOSITY OF BLOOD IN TERMS OF SERUM PROTEIN AND HEMATOCRIT  
 $H = 1/(1 - \text{hematocrit})$ .

DISCUSSION

The propriety of a mathematical treatment of renal resistance to blood flow can be questioned at the present time because of its dependence on "certain absolute values" to obtain a quantitative expression (8). Such criticism is fundamentally unsound when it is used to support

instead a non-mathematical treatment. The non-mathematical (or dialectic) method is concerned with the very same controlling quantities as the mathematical ones, but can achieve what appears to be a wider range of application and less dependence on these "absolute values" only by implicit neglect of the limitations which are

inherent in the basic renal relationships. By stating premises concretely and limiting them where necessary, reasoning along mathematical lines can offer results, the underlying postulates of which are clear. A literary treatment simply obscures these facts and can lead to incorrect conclusions (9, 11, 12).

The special virtue of algebraic expressions is the neat way in which the influences of many variables can simultaneously be permitted to interreact. Our minds, unaided by such tools, cannot be expected to assess the relative influence on resistance of changes in blood viscosity, blood pressure, blood flows, and so on. Far from assuming fixed afferent and efferent arteriolar resistances (8), wide variability of both is to be expected and obtained; the mode of derivation of the formulas does not presume fixity. This was clearly indicated in the initial application of these methods to renal resistance in man during spinal anesthesia, where changes of both sets of arterioles were routinely seen (1).

Nonetheless, because of the formulas' flexibility, and because it had previously been reported that in man, during the change from renal ischemia due to adrenalin to the renal hyperemia induced by pyrogens, the main shifts are in the efferent arterioles alone (3), it was deemed advisable to determine the effects of a fixed afferent or efferent set of arterioles. Our study indicated that, in this physiological range, both sets of arterioles, rather than only one, participated (11).

"The glomerular filtration rate cannot in theory, or in practice, approach zero at high renal blood flows . . .," if the afferent arterioles and systemic blood pressure are fixed, according to Shannon (8). Yet, if we visualize our ideal nephron in this condition, we can see that, as efferent arteriolar resistance is progressively reduced, the drop in blood pressure along the efferent arterioles from glomerulus to peritubular capillaries must also progressively decrease, while blood flow, because of the consequent reduction in total renal resistance, rises. That is to say, as blood flow increases, glomerular intra-capillary pressure falls until, if carried to a theoretical (not physiological) extreme, it will be the same as peritubular capillary pressure and will be inadequate to form a glomerular filtrate. This is the meaning of the extreme foot of the curve pre-

viously reported (11). It is not meant to be a physiological occurrence, since our formulas were carefully limited to physiological ranges so as to prevent drawing such impractical conclusions. We thus see that our ideal nephron, when carried past its well defined range, works reasonably, although ceasing to be applicable to biological problems.

That the calibre of arterioles, and therefore their resistance, varies with the pressure of the blood inside them (8) does not interfere with our deductions. Our "individual resistance," elaborated above, refers to the resistance to flow of the individual's own blood, and imposes no restrictions on the response of the arteriole to the pressure of the blood within it. "Hydraulic resistance," as we have used it, permits some conclusions concerning the relative calibre of the vessels, but again without neglect of their response to internal pressure.

#### SUMMARY

The similarity between blood flow and the flow of plastic solids, as described by Bingham, is used to substitute a variable in place of a static value for the yield pressure in our application of Poiseuille's Law to the kidney.

An expression for post-arteriolar resistance (defined precisely) is added to those for arteriolar resistance so that total renal resistance to blood flow can be computed, if desired.

A method is elaborated by which serum protein of any albumin-globulin ratio can be quickly converted by means of a chart (or formula) into an osmotically equivalent concentration of serum protein of A : G ratio 2.20.

A second chart then provides the systemic osmotic pressure of blood (or plasma) for the osmotically equivalent concentration of standard serum protein and also the osmotic pressure of the blood concentrated in the glomerulus by ultrafiltration to the degree indicated by the inulin-to-diodrast clearance ratio. These two values simplify and improve the calculation of renal resistance to blood flow.

A chart for blood viscosity, as affected by the serum protein concentration and the hematocrit, permits the ready calculation of renal resistance with allowance for the viscosity of the subject's blood.

While the charts for osmotic pressure and viscosity of blood have been designed primarily to aid in studying renal function, they have wider applicability wherever blood viscosity and osmotic pressure are required without direct measurement.

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# THE RELATIONSHIP OF DEHYDRATION AND OVERHYDRATION OF THE BLOOD PLASMA TO COLLAPSE IN THE MANAGEMENT OF ARTIFICIAL FEVER THERAPY<sup>1</sup>

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The most common serious reaction encountered in the conduct of artificially produced fever therapy is a state of collapse which may appear at any time during the treatment. In 1100 treatments given in our clinic between 1930 and 1937, there was a 1 per cent mortality, and many other serious collapse reactions which were in some way concerned with the water and salt excretion and intake during the treatment. Some of the patients in the early period lost from 3 to 5 kgm. in body weight during short treatments of 5 hours, while others lost little or no weight during prolonged treatments (10 to 27 hours) at 41.5° C. Later in this period, when the water intake was better adjusted and sodium chloride administration was included during treatment, the loss in weight was reduced, and the collapse state was less often encountered. Yet it still occurred at odd times in the year and in a curious sequence of cases. It bore no relation to the disease for which the patient was being treated nor to the length of treatment administered, and often occurred in what ordinarily would be termed a "perfectly healthy young adult."

This collapse state has many of the "ear marks" of what is termed surgical or traumatic shock in that there is an absence of sweating and urine, pallor, cold extremities even with rectal temperatures of 41.5° C. or higher, fall in blood pressure and venous pressure, retardation of the skin capillary response to digital pressure, cyanosis, and coma. Fatal outcome may be precipitated by a maniacal state before the coma ensues. Caffeine and adrenalin are ineffective, and oxygen-carbon dioxide mixtures are only of temporary benefit. In the early stages, vomiting prevents the ingestion of water, although concentrated 2 to 25 per

cent saline is occasionally tolerated and may resuscitate the patient. One or more liters of physiological saline injected intramuscularly are usually promptly effective if the collapse state is not too far advanced to prevent its absorption. In such a case, an intravenous injection of this volume must be given and injected slowly. Any intravenous technique for therapeutic administration or chemical data is a difficult maneuver when the information is most desired because the veins are usually collapsed or constricted in the collapse state. Furthermore, any manipulation excites the patient, with a resulting rise in temperature which further increases the precariousness of his situation. This hypersensitivity of the patient has prevented to a great extent any extensive study of his metabolic state. Thus, most data have to be obtained in almost a hit or miss fashion, as opportunity offers.

Alterations of the red blood cell count, hemoglobin (1 to 4), and hematocrit, while indicative of hemoconcentration, do not change significantly (greater than 10 per cent) until dehydration is well advanced. Other factors may influence these values, thus rendering them of little aid in the control of fever therapy cases (2, 5 to 9). Determination of the circulating blood volume (10) or the extravascular water volume (11) during a routine treatment is not a simple procedure and cannot be repeated at will.

The work of Gibson and co-workers (12, 13) has shown that a definite decrease of approximately 15 per cent in the circulating blood volume occurs during the induction of artificial fever when sweating is at its maximum. This volume may be decreased still further with resultant hemoconcentration if the water and electrolyte balance is not restored as treatment progresses (1, 14 to 21).

<sup>1</sup> Assisted by grants in aid from the United States Public Health Service and the Rockefeller Foundation.

From this brief summary, it can be seen that the uniformly safe performance of prolonged artificial fever at high temperatures has definite possibilities if some simple test can be found to indicate which patients are bordering on a precarious state, and likewise to indicate when the proper readjustments to the differing requirements of each patient have to be made. Also, it should be possible to so design the treatment procedure that all of the adjustments of fluid and electrolyte balance can be made by ingestion rather than by injection.

Data accumulated between 1935 and 1939 from 50 cases (Table I), with and without reactions, indicate that while the plasma chloride values did not change much (1 to 10 per cent), the values were as apt to rise as they were to fall during the time of the reaction. Furthermore, there was no direct correlation between the chloride values and

the change, if any, in the total serum protein. Significant, however, was a striking and constant diminution in the chloride content of the urine. This was first evident in the urine passed during the treatment, for the last urine passed before a collapse state ensued contained little and occasionally no demonstrable chloride. Furthermore, the total urine output for the day or two after treatment was always low in chloride content. Not infrequently, the first 24-hour volume (500 to 2500 cc.) might yield only  $\frac{1}{4}$  to  $\frac{1}{2}$  gram of chloride, and several days might pass before this rose to 5 or 10 grams for the 24-hour period. The blood chloride was frequently 10 to 30 per cent below normal for a day or so after treatment when collapse had occurred. During this short interval, the patient also regained most of the weight lost during treatment unless vomiting complicated the picture. If 15 grams of sodium chlo-

TABLE I

*Characteristic exchanges during treatment at 41.5° C. before the water and sodium chloride intake was properly adjusted (1935-1938)*

Samples were taken just before treatment and at the end of treatment while the patient was still at a temperature of 41.5° C. These were all patients in excellent physical condition.

Patients	At 41.5° C.	Total serum proteins		Blood chlorides		Fluid intake	Urine during treatment	NaCl intake	Urine NaCl	Reaction
		Start	End	Start	End					
	hours	grams per 100 cc.		m.eq. per L.		cc.	cc.	grams	grams	
Mil	3	6.9	8.5	102	101	1800	60	0.0	0.0	Severe collapse; stopped treatment.
	15	6.4	6.9	102	111	3600	40	17.5	0.3	3rd hour, TP=8.5; 5 grams of NaCl p.o. stopped collapse.
Fel	13	6.7	6.3	93	104	4000	355	7.5	1.4	None.
Wei	16	6.2	6.7	103	95	3200	130	0.0	0.3	None.
Du	11	6.3	7.4	98	98	2700	0	0.0	0.0	Collapse; treatment stopped.
Geb	8	6.6	7.4	103	99	2200	100	0.0	3.0	No sweating at end of treatment. Otherwise uneventful.
Hut	11	7.1	7.2	92	109	2400	228	20.0	0.4	Vomiting, dry skin. Otherwise uneventful.
McB	13	7.4	7.2	97	98	3800	40	5.0	0.1	Restless; dry skin. Otherwise no reaction.
Dir	18	5.9	7.3	99	96	3500	220	0.0	0.6	0.6 grams NaCl in 20 cc. vomitus. Dry skin.
Pal	14	6.7	7.3	101	100	3400	5	5.0	0.0+	Dry skin; low blood pressure; jaundice; restless.
Col	5		5.9	100	107	1800	190	0.0	0.6	None.
Leng	14	6.2	6.4	99	102	3600	520	7.5	2.5	None.
Ga	15	6.7	6.4	103	105	3600	1075	7.5	1.3	None.
Ger	16	5.9	6.8	104	101	3700	100	10.0	0.2	Skin slightly dry. No other reaction.
Pet	16	6.8	7.2	104	97	2800	130	7.0	1.2	Restless. Otherwise uneventful.
Slo	15	5.6	5.9	105	110	3300	25	15.0	0.2	Restless; dry skin.
Ro	17	6.8	7.1	88	91	4050	140	7.0	0.4	Restless; dry skin; low blood pressure. Otherwise none.
Bi	16	6.7	6.7	106		4000	734	10.0	4.1	None.
Skö	21	6.0	6.6	103	106	5100	480	15.0	3.3	None.
McGo	22	5.7	6.1	104	103	4350	430	10.0	3.8	None.
Vic	27	6.2	5.8	97	108	6200	1688	20.0	7.2	None.
DFa	16	6.4	6.5	96	91	3000	100	5.0	0.1	0.8 grams in 400 cc. vomitus. Skin dry at end of treatment.
RFa	16	6.6	6.9	101	105	4000	1080	10.0	2.3	Restless; slight jaundice.
Al	14	6.8	7.4	84	89					
Creid	14	6.4	6.4	98	106	3600	280	10.0	1.1	Pallor but no other reaction.

ride were given daily to supplement that in the diet, only about 5 grams would appear in the first and second days' output, and not until the third or fourth day would the output approach or pass the 15 gram excess of sodium chloride administered.

It soon became apparent that the empirical administration of 1 gram of sodium chloride plus 500 to 600 cc. of water per hour by mouth was approximately the basic requirement for the proper management of the average patient at 41.5° C. for periods of 10 to 20 hours' duration, and that additional sodium chloride must be given following treatment. Reactions became less frequent on this routine but occasionally occurred when least expected.

A survey of the total protein changes in the 50 cases mentioned above showed (Table I) that there was a good deal of consistency between the rise in serum protein values and the occurrence of reactions bordering on collapse. Cases showing serum protein values of 7.0 grams or lower usually did well. Those with values above 7.0 grams usually showed some symptoms of the collapse syndrome. Occasionally, a patient with serum protein values of 8.0 grams at the end of treatment did well, yet he might show a severe chloride deficit in his urine during the succeeding days. The rise in serum protein seems to be a result of reduction of plasma volume following the obviously large and rapid losses of electrolytes and water as a result of profuse sweating (22, 23), rather than a gain in circulating protein from the body stores (24, 25) although some exchange can readily occur. It is difficult for us to believe that a negative nitrogen balance (26) occurs during the febrile state. Without evidence to the contrary, it is probable that the total quantity of circulating plasma protein remains fairly constant (27, 24, 25) during the treatment period. The plasma chloride and water, on the other hand, are subject to tremendous exchange through ingestion, excretion (sweat and urine), and exchange with the extracellular spaces (23, 37). Total protein values indicate at least the trend of concentration of the blood, and over the short period required are thus a relative measure of what is happening to the circulating plasma volume. In turn, this is probably a reflection to some extent

of the state of the interstitial store of fluid and electrolytes (28 to 30).

The determination of the total protein is too unwieldy a procedure to be used as a quick, simple guide while treatment is underway and quick decisions must be made. The plasma specific gravity technique, done by the pyknometer gravimetric method (31), on the other hand, is simple, rapid (10 to 15 minutes), and requires little blood.

The protein content has the greatest influence upon the specific weight of the plasma or serum. During the treatment, total protein elevations of 15 per cent (from 5.9 to 6.8 grams) will occur, and the chloride will change about 2 per cent (104 to 101 m. eq./L.), or less than the accuracy of both the chemical and specific gravity methods. This protein change represents a change in plasma specific gravity from 1.0243 to 1.0270.

Plasma specific gravity data have been consistently taken for the last 5 years and over 4500 determinations give a good catalogue of the normal and abnormal states encountered (Tables II and III). The ranges in Table I are similar to those reported by Moore and Van Slyke (32). We have now had enough experience in the interpretation of these data to use plasma specific gravity as the principle guide to the adjustment of the fluid and salt requirements of the patient during our routine treatments of 15 hours at 41.5° C. Occasional individual and unexplainable variations may be encountered so that one must proceed with the treatment more cautiously in such cases.

TABLE II

Hydration status	Plasma specific gravity
Dehydration	Greater than 1.0290
Normal	1.0255 to 1.0290
Low	1.0233 to 1.0255
Overhydration	
Edema zone	1.0225 to 1.0233
Edema	Less than 1.0225

Judging by the plasma specific gravity standard, many patients are admitted to the hospital with their fluid stores near depletion. Failure to replenish this store by the administration of salt and water before treatment has resulted in the past in many cases of dehydration, even during short fever treatments. Therefore, in preparing a patient for fever treatment, it is advantageous to give 12 to 15 grams of salt plus fluid *ad lib.* (3 to 4 liters) to the patient on the day before



TABLE III

*Characteristic changes in the plasma specific gravity*

Most of these patients were in bad physical condition and the worst risks are marked with an (\*). They were treated at a lower temperature of 40.5° to 41.0° C. because of this. Extrapolations are from Peters and Van Slyke (33).

Patients	Treated at 41.5° C.	Plasma specific gravity			Extrapolated total proteins			Blood chloride			Reaction
		Start	5 hours	End	Start	5 hours	End	Start	5 hours	End	
	<i>hours</i>				<i>grams per 100 cc.</i>			<i>m. eq. per L.</i>			
Cal	10	1.0280	1.0253	1.0328	7.2	6.4	8.2	106	102	106	+++
Mais	6*	1.0243	1.0263	1.0268	5.9	6.4	6.6	104	103	104	+
	10	1.0247	1.0258	1.0262	6.0	6.4	6.6	101	100	97	+-
Bo	9*	1.0278	1.0288	1.0270	7.1	7.4	6.8	102	99	99	+
	10	1.0254	1.0250	1.0251	6.2	6.1	6.2	103	101	98	+-
Sar	10	1.0291	1.0328	1.0300	7.4	8.5	7.9	105	102	100	+-
Go	10*	1.0254	1.0283	1.0278	6.3	7.2	7.0	108	105	106	+
	11	1.0265	1.0271	1.0293	6.7	6.9	7.6	111	106	107	+-
Ko	11	1.0271	1.0275	1.0285	6.9	7.0	7.4	105	101	100	+-
Ha	11	1.0284	1.0284	1.0284	7.4	7.5	7.4	103	100	100	0
Mu	5*	1.0262	1.0260		6.6	6.5		103	99		0
Sin	10	1.0298	1.0276	1.0294	7.7	7.1	7.9	114	110	91	+-
Moul	15	1.0269	1.0271	1.0281	6.8	6.9	7.1	110	108	104	+-

0 = no reaction.

+- = dry skin, moderately low blood pressure (80 to 70 mm. systolic) at end of treatment.

+ = restlessness, dry skin, low blood pressure, mild prostration.

+++ = collapse as treatment ended; recovered, slight jaundice.

All of these patients were cases of advanced paresis with a bad prognosis, and either would not have been accepted for treatment previous to 1941 because of the bad risk or would have been treated with the knowledge gained from previous experience that one or more deaths would ensue from collapse. The preliminary treatment at the lower temperature improved them enough to enable three of them (\*) to tolerate the full treatment of 10 hours at 41.5° C. later.

treatment. Most of this water must be stored since the patient usually gains from 3 to 4 kilograms in weight over night, and the plasma specific gravity reading may drop 10 to 20 per cent. Longer periods of salt administration may produce catharsis and gastric irritation, or even some actual overhydration which may be accentuated during treatment unless fluids and salt are later restricted. Occasionally, the administration of 15 grams of salt and appropriate amounts of water over a 3-day period previous to treatment has resulted in a diuresis at the end of this time which has depleted the patient, just before treatment, of more water and salt than was stored. Thus, some patients were near overhydration at the start of treatment and others were actually dehydrated. Prolonged treatments at 41.5° C. are not feasible as a routine procedure without properly "filling" the body's store with salt and water, yet when the adjustment is properly done, such long treatments may be accomplished with surprising ease and safety.

Originally, plasma specific gravity measurements were made daily for 3 to 4 days prior to and following treatment, and as often as hourly during the course of the fever. Now, however,

after one day of salt administration, usually of 15 grams, a determination is made immediately prior to the induction of a prolonged fever of 15 hours at 41.5° C., and another 5 to 7 hours after the beginning of treatment, and a third at the close of treatment. These 3 determinations are usually sufficient. If there is any abnormal change in the patient during or after fever, plasma specific gravity measurements are made as required.

During the process of treatment itself, 400 to 500 cc. of heavily sugared weak tea, dilute fruit juice or water are given per hour plus 1 gram of salt in 100 cc. of water per hour for the first 4 to 5 hours. Throughout the rest of the treatment, fluids are given at about the above rate but 1 gram of salt is given every other hour or not at all, as indicated by the plasma specific gravity readings. Unless otherwise indicated, all of the fluids and salt are given by mouth.

During the past 48 months, up to July 1942, 406 prolonged treatments employing the "radiant energy" cabinet have been guided by the plasma specific gravity measurements as determined by the pipette pyknometer method (31). In the last 100 fever treatments of 10 to 27 hours at 41.5° C. done just previous to 1939, there was one death,

and approximately 28 per cent of the cases displayed dehydration severe enough to give clinical signs and rises of 15 to 30 per cent in the total protein values. In the next 100 cases given 10 to 18 hours of fever at 41.5° C. during 1939 to 1940, while we were adjusting the fluid and salt intake in accordance with the plasma specific gravity values, there was one death (reported below) and approximately 20 per cent of the cases were to some degree dehydrated. Between 1940 and July 1942, however, over 300 cases have been treated with less than 10 per cent of them falling in the dehydration range. There were no mortalities in this group. Many of these patients were paretics in such poor physical condition that they would not have been accepted for treatment previous to 1940 (Table III). In some instances, the administration of fluid has been overdone and a new syndrome of overhydration produced which, in many respects, causes symptoms resembling dehydration.

During the period of fever treatment it has been found that the patient's body fluid status, as indicated by the plasma specific gravity readings, may be divided into any one of 3 general groups:

1. The plasma specific gravity level may start at and be maintained well within the normal range of 1.0255 to 1.0290 by the proper fluid and salt administration.

2. The specific gravity levels may be already high or may rise during treatment, indicating that dehydration is present. This must be corrected or the treatment stopped if this trend persists or increases.

3. The plasma specific gravity may fall from the initial value, indicating plasma dilution. This may represent adequate or excessive fluid storage, in which situation, fluid and salt should be restricted.

Characteristic illustrations of these variations are presented in 3 classifications: (1) the well controlled or satisfactory group; (2) the dehydration group; and (3) the overhydration group.

#### 1. SATISFACTORY GROUP

The criteria for classification within this group are: (1) tolerance of the patient to the therapy, with no marked irregularities of pulse, respiration, or blood pressure, and complete recovery by

the following day. In cases treated routinely for periods varying from 5 to 18 hours at 41.5° C., the respirations may be from 20 to 40 per minute, the pulse 140 to 160 per minute. During the induction and the first hour of fever, the systolic blood pressure may rise 20 to 40 mm. Hg higher than at the start of treatment. During the latter part of therapy, the systolic pressure may fall to 100 or 120 mm. Hg and rarely goes lower than 20 mm. below the patient's normal blood pressure level. (2) Nausea, vomiting, or diarrhea are absent. The output of urine containing some sodium chloride continues at regular intervals. There is a "normal" amount of sweating although some patients sweat more profusely than others. Neither age, the disease for which the patient is being treated, nor any other obvious reasons seem to be related to the amount of sweating present. (3) The patient is able to sleep during most of the treatment without narcosis and with a minimum of restlessness when awake. (4) The plasma specific gravity is maintained in the normal range of 1.0255 to 1.0290. (5) In this group there were 369 treatments, or 91 per cent of the entire series.

Four selected cases have been chosen to demonstrate the various changes in plasma specific gravity which may occur during a satisfactory fever treatment at high temperatures. The large although variable maintenance requirements of both salt and water are of particular significance in estimating the needs of a case in order to prevent symptoms of advancing dehydration from any cause. The gain or loss in body weight, if any, can be about 2 kgm. without causing clinical symptoms or deflecting the plasma specific gravity out of the normal range. This weight loss is readjusted by the patient within a few days following treatment. The urine excretion varies between  $\frac{1}{4}$  and  $\frac{1}{7}$  of the fluid intake. The rôle of the sweat glands as an excretory organ of high order during fever thus becomes apparent.

Before the advent of sulfanilamide, fevers of 15 to 27 hours' duration at 41.5° C. were not unusual. A single prolonged fever is still often given for the treatment of refractory cases of gonorrhea (32, 34), and is also being used in our clinic for the treatment of paresis, whenever feasible. Four selected cases of rheumatoid arthritis were given experimental 48-hour treatments at a temperature of 39.5° C. In these cases, where

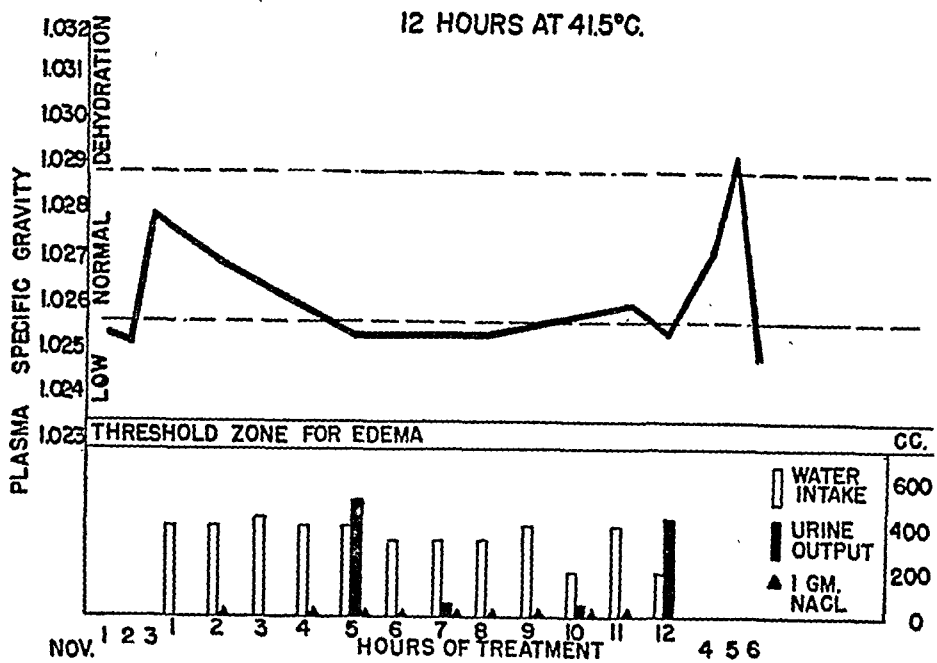


FIG. 1

sweat loss is of such great magnitude, it is especially important that the control of fluids and electrolytes be adequately and safely guided.

Case Number 1 (Figure 1): This case illustrates a routine 12-hour treatment, at a rectal temperature of 41.5° C., of a 40-year-old woman with latent syphilis. The patient had an intake of 4220 cc. of fluid and 10 grams of salt, with a urine output of 630 cc. Blood pressure varied but slightly from its original level (average pulse 120, respirations 27). Interesting to note is the overnight rise of specific gravity between November 2 and November 3 (the day of treatment). This has frequently occurred and is probably due to excessive kidney excretion as a result of nervousness in an apprehensive patient at the prospect of

treatment the next day. With adequate administration of fluid during the first few hours of treatment, the plasma specific gravity was soon brought down to the optimum level where it remained throughout the treatment. The patient tolerated the therapy extremely well but for one small emesis, some nervousness and apprehension. Her condition at the end of treatment was excellent. The rise and fall of her plasma specific gravity during the 3 days following treatment is commonly found and probably represents an overcompensation during readjustment of the water balance mechanism which has been subjected to a severe strain by the fever.

Case Number 2 (Figure 2): This patient was a 52-year-old parietic who was treated for 10 hours at a temperature of 41.5° C. The plasma specific gravity level the

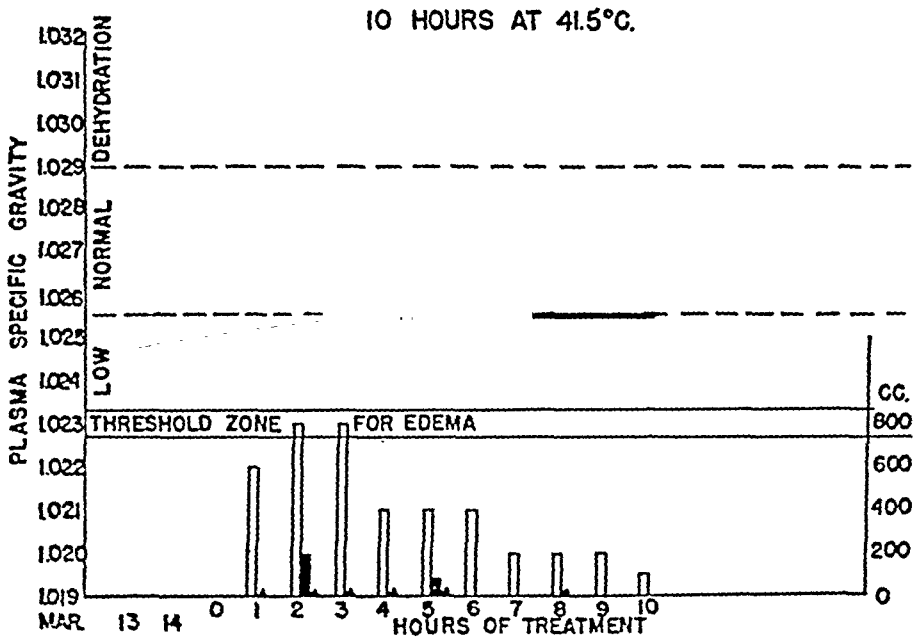


FIG. 2

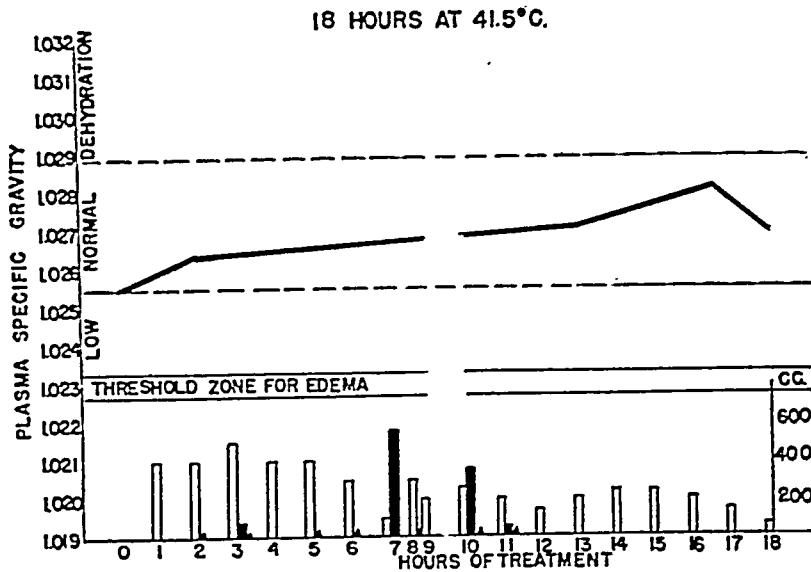


FIG. 3

day prior to treatment showed that he had stored water in response to the prefever hydration regime. There was little change from this level overnight. Since most of the patients showed a tendency to dehydrate during the first few hours of fever, fluids were forced during this period. At the third hour, the plasma specific gravity level was still optimum so gradually the fluids were reduced until it was found that the plasma specific gravity levels at the 8th and 10th hours could be maintained by only 200 cc. of fluid per hour and 1 gram of salt. His total fluid intake was 4500 cc. and 6 grams of salt were given. Urinary output was 280 cc. He perspired markedly throughout treatment. His weight at the start of treatment was 92.6 kgm. and at the end was 89.3 kgm. His average pulse and respirations were 100 and 32, respectively. Blood pressure showed a gradual downward trend from 150/76 to 90/46 at the close of treatment. The patient tolerated the fever well and his post-fever course was uneventful.

Case Number 3 (Figure 3): This chart illustrates the satisfactory treatment of a 43-year-old male, treated for gonorrheal arthritis at 41.5° C. for 18 hours. His total fluid intake was 4700 cc. and he received 14 grams of salt. His urinary output was 900 cc. and he vomited a total of about 900 cc. in small amounts at various time intervals, reducing his actual intake to 3600 cc. Sweating was intense. His clinical status remained within normal limits. It may be seen from the chart that there is a slow gradual tendency for his plasma specific gravity to rise, yet it did not reach a high level at any time. He was adequately prepared, had stored fluid well, and with the administration of nearly the proper amount of fluid and salt during the treatment, his dehydration did not approach a dangerous level. His clinical condition at the close of treatment was good.

Case Number 4 (Figure 4): A 48-year-old woman with infectious arthritis was given a 48-hour treatment at

39.5° C. Her intake over this period was 13,840 cc. of fluid and 29 grams of salt. The urinary output was 5460 cc. and there was no emesis. This case was very well controlled. Water and salt intake was reduced after 26 hours of treatment, and the plasma specific gravity rose to 1.0278 by the 34th hour. The fluids were therefore proportionally increased and a corresponding fall in plasma specific gravity was noted. The specific gravity level during the last 8 hours was near the optimum of 1.0255. That she was well hydrated and the interstitial reservoirs well filled was indicated not only by the plasma specific gravity levels but also by the continued sweating, urine output, and a normal blood pressure, and no weight loss. Her reactions are typical of 3 others similarly treated.

## 2. DEHYDRATION GROUP

The criteria for classification in this group are: (1) The psychic intolerance of the patient to therapy, which increases dangerously if the dehydration state persists. Depending somewhat upon the rate of development of the dehydration state, the urine output diminishes and then ceases. Sweating stops. The patient may show fluctuations in the rate of pulse, respiration, and blood pressure. (2) If the dehydration is severe, the respirations increase and become shallow, cyanosis appears, the pulse rises to 160 or 170, and the systolic pressure begins to fall rather rapidly. The pulse pressure increases and may finally disappear. The skin is bluish and often cold. The extremities are cold, even with a rectal temperature of 41.5° to 42.5° C. (3) Nausea may be present but only after the patient has over-com-

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48 HOURS AT 39.5°C.

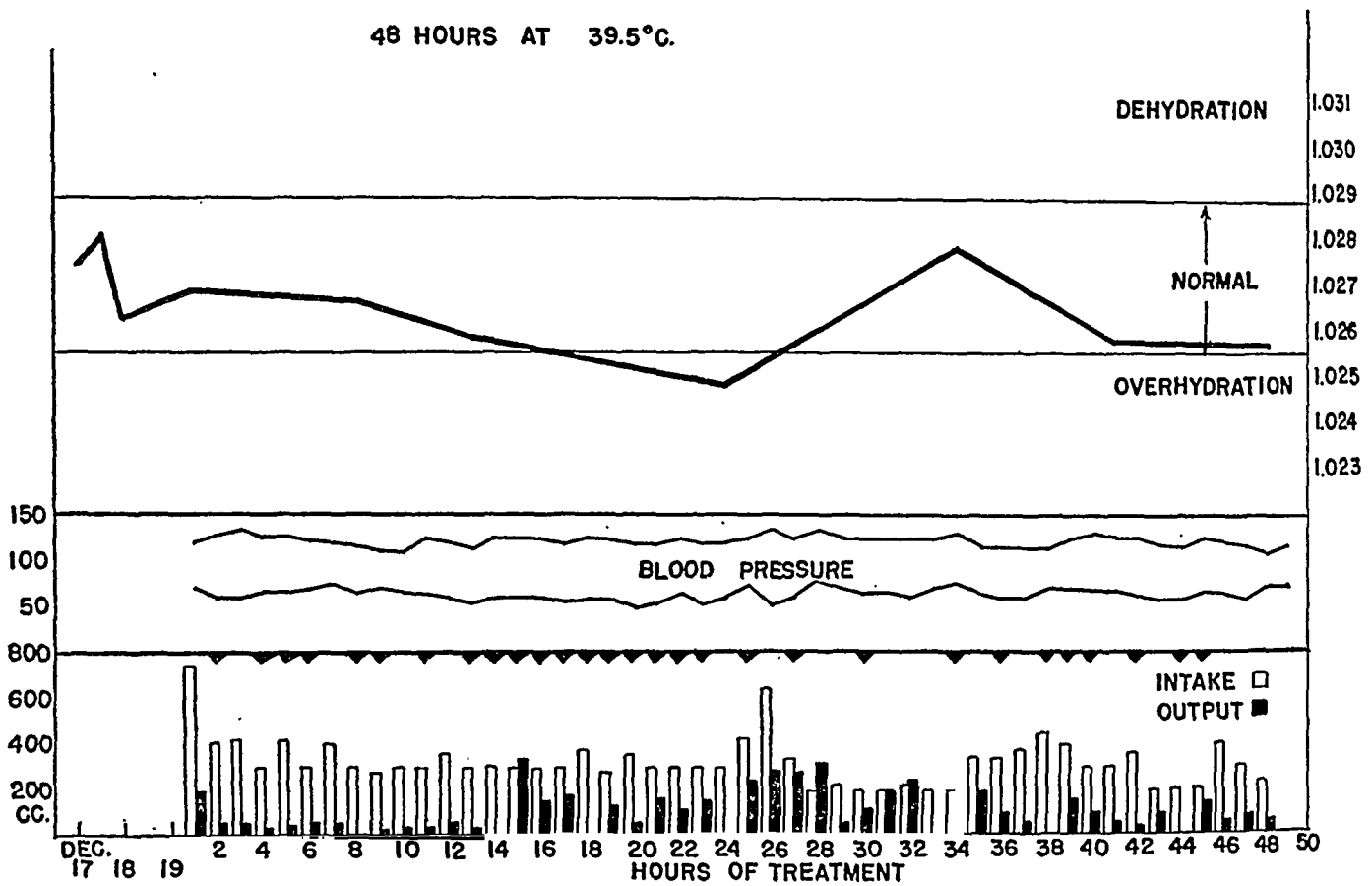


FIG. 4

pensated his thirst by the amount of fluid without salt that the stomach can care for. The emesis contains little or no chloride. (4) Extreme restlessness ensues and maniacal trends develop to the time of unconsciousness when the collapse or shock state becomes fully evident. Maniacal states are accompanied by physical exertion which is accompanied by a rise in temperature. It usually defeats attempts to cool the patient down. Such maniacal states are now controlled by the use of 0.5 gram to 2.0 grams of Evipal intramuscularly or intravenously, which allows us to reduce the temperature before a dehydration collapse ensues yet still permits the patient to recover consciousness rapidly enough to cooperate in taking fluids by mouth. In Case Number 5, the clinical signs of collapse came on too rapidly during the maniacal state to alter the course of the treatment in time. Had we had more experience with the plasma specific gravity method at that time, the development of the dangerous dehydration state would have been appreciated earlier and precautions would have been taken to prevent it. (5) When the clinical evidences of dehydration

are present, the plasma specific gravity has always been in the dehydration range above 1.0290. In the average case, a change to the high normal specific gravity level, or a rise from normal into the dehydration zone, indicates the possible onset of dehydration well before there is any clinical evidence for it. (6) There have been situations, however, when treatment has been continued even with the specific gravity reading in the dehydration range throughout the entire course of treatment. In such cases (Numbers 7 and 8), the situation was apparently otherwise normal since sweating and urine output continued, and the clinical status was within the normal range. This is in some way related to a seasonal variation which will be discussed later in this paper. (7) A prolonged period of inadequate supply of salt and fluid at these high temperatures in the presence of dehydration may also result later in the appearance of jaundice which is apparently due to damage to the liver. The jaundice may be severe and add days to the hospitalization of the patient. This is now rarely seen since the fluid and salt balance have been controlled. Jaundice is little

affected by carbohydrate administration alone, but responds more rapidly to adequate saline administration of from 15 to 20 grams daily plus plasma protein transfusions. (8) Recovery from advanced dehydration states may take from 3 to 10 days, even with adequate water and salt administration. The symptoms are anorexia, vomiting, malaise, and occasionally anuria and fever. The loss of weight may be as much as 4 to 6 kgm. (9) In this group, there were 28 treatments, or 6 per cent of the entire series of cases. Three selected cases have been chosen to illustrate the various changes in plasma specific gravity, etc., which occur in the state of dehydration.

Case Number 5 (Figure 5): One of the early cases in this series demonstrates quite well how rapidly dehydration may develop with little warning and progress to a fatal termination with collapse or shock. This patient was a 41-year-old obese male, in poor physical condition, who was treated at 41.5° C. for advanced central nervous system syphilis. The fluid intake was not guided by plasma specific gravity measurements at this time, and it is noted that there was actually very little change in his level during the prefever stage, showing that he had probably not increased his fluid reserve. Overnight before treatment, the plasma specific gravity level rose from 1.0264 to 1.0278 (upper normal range). Thus, when fever was started the profuse sweating which ensued soon depleted his fluid and salt stores to a dangerous level. Clinically, he seemed to be doing poorly from the onset of therapy although his blood pressure was maintained satisfactorily. Two hours after the start of therapy his temperature was 41.5° C., and shortly afterward the patient

developed an uncontrollable mania. His condition rapidly became critical with ensuing collapse and coma. Up to that point he had received 900 cc. of fluid and 1 gram of salt, the usual amount. There was then an emesis of 100 cc. followed by a sudden and complete cessation of sweating. The pulse became barely perceptible and the blood pressure could not be obtained. He was given 500 cc. of Ringer's solution subcutaneously and 1000 cc. intravenously but he expired 2 hours later, 7 hours after the start of treatment, and despite all efforts to revive him. At the 4th hour (even after some of the infusion fluid had been given), the plasma specific gravity was 1.0327. The collapse of this man was obviously precipitated by the onset of his maniacal state while he was in the lower and early stages of dehydration. Post mortem examination revealed no definite cause of death.

Cases Number 6 and 7 (Figures 6 and 7): In February 1939, 2 cases were treated on the same day. Both were young men in good physical condition. They had both appeared in the Syphilis Clinic on the same day with acute primary and secondary lesions of syphilis. Both had temperatures varying from 38.0° C. to 38.5° C. and appeared moderately acutely ill. It was decided to treat them with fever, neither having had any chemotherapy. The day previous to treatment it was found that the plasma specific gravity levels of both men were in the high level for the normal range. Fluids were not well stored, probably because of their febrile condition and also perhaps because of the seasonal factor.

At about the time their induced fevers had reached 41.5° C., both patients showed what were ordinarily considered to be dangerously high plasma specific gravity levels. There was an immediate increase in the administration of fluid and salt for both patients. "S" responded fairly well clinically, but "R" failed to accept his fluids properly so the cabinet was opened at the 4th hour and

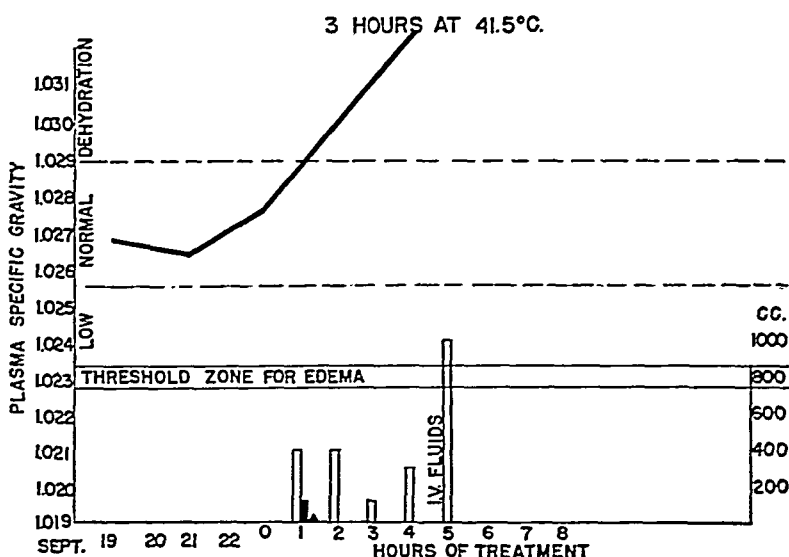


FIG. 5

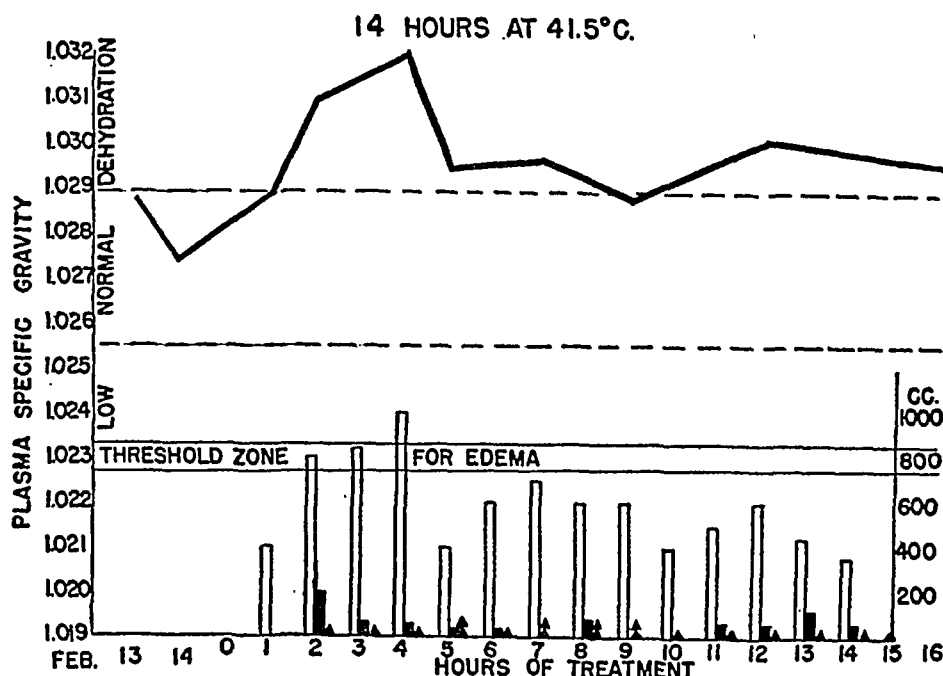


FIG. 6

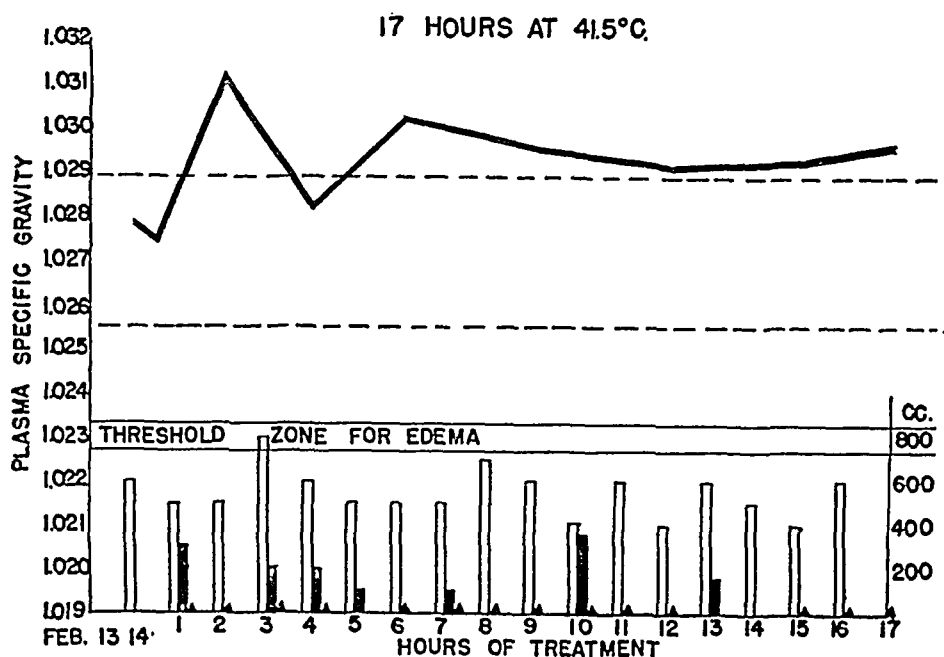


FIG. 7

his temperature brought down slowly. After a brief time, his clinical condition improved and the treatment was resumed at 40.5° C. Having passed this critical period, both cases continued to receive their treatment, "R" for a total of 14 hours, and "S" for 17 hours, at 41.5° C. The plasma specific gravity readings for both continued just above the normal range during this period. This would seem to indicate that their interstitial reservoirs were apparently near depletion, yet both of these patients had profuse sweating and appeared to react normally. "R" had a total intake of 9050 cc. and 17.5 grams of salt, with a urinary output of 950 cc. His blood pressure, pulse, and respirations were well sustained. Toward the

end of treatment, he ceased sweating and treatment was stopped. He recovered promptly from the treatment without sequelae. "S" had a total intake of 8700 cc. and 16 grams of salt with a urinary output of 1185 cc. His clinical status also showed little variation from the normal. These cases indicate that even though undesirable levels are present, treatment may progress with caution as long as the plasma specific gravity levels are carefully followed.

Since these 2 cases not only continued to sweat but also had rather large volumes of urinary output, we were at a loss to explain fully their good condition at such high specific gravity levels. Assuming that a seasonal shift in

the plasma specific gravity level had occurred, as suggested above, it may be that these patients were actually maintained on a narrower fluid reservoir margin at this period of the year. These 2 cases are good examples of several which have occurred during this period of the year.

### 3. OVERHYDRATION GROUP

The criteria for classification in this group are: (1) The psychic intolerance of the patient to therapy as long as the overhydration state persists. Irregularities of pulse, respiration, and blood pressure are noted. The respiration rate may increase and often becomes stertorous. The pulse may be rapid (120 to 160 per minute). The blood pressure may first rise to hypertensive levels and then as collapse ensues, it may fall below 80 mm. Hg systolic. (2) Nausea and vomiting appear rather early. Diarrhea may also be present. Strangely enough, the urine is first as copious as is the diaphoresis but as the overhydration state becomes more extreme, the renal mechanism fails and sweating ceases. The skin may be very dry, with no evidence of perspiration in any area. The extremities are cold and blue, and the patient actually appears dehydrated. (3) Early in the overhydration state, there is no evidence of gross edema. As the state becomes more severe, however, puffiness is noted about the face and eyes, and gross edema appears which may even become general, involving the ankles, legs, sacrum, and arms. (4) The patient becomes rest-

less, irritable, and emotionally unstable. Later, muscular twitchings, athetoid movements of the arms and legs (not tetany) may appear. In the advanced states of overhydration, severe and almost continuous grand mal seizures are present. It may be necessary to administer intravenous Evipal to stop these serious seizures, as is done for the mania accompanying dehydration states. The body temperature should be reduced as quickly as possible, since below 40° C. these central nervous system phenomena usually disappear without the aid of anesthesia. Recovery after treatment is slow, for the patient is in a "water logged" state. Mental confusion and irritability often last for 2 to 7 days. This is frequently accompanied by a spontaneous fever of several degrees. These mental symptoms have not been observed in patients who are dehydrated. (5) The gain in weight may be as much as 5 or 6 kgm. which are later lost by diuresis. (6) The plasma specific gravity measurements in these cases of overhydration are below 1.0245. A rapid drop from high normal to this level may present some symptoms. The plasma specific gravity trends show very early any tendency to overhydration. Thus, the serious overloading of the system with salt and water may be avoided. This measurement is of great aid in differentiating between overhydration collapse and dehydration collapse. In fact, in cases where sweating is absent, this may be the only means of differentiating between

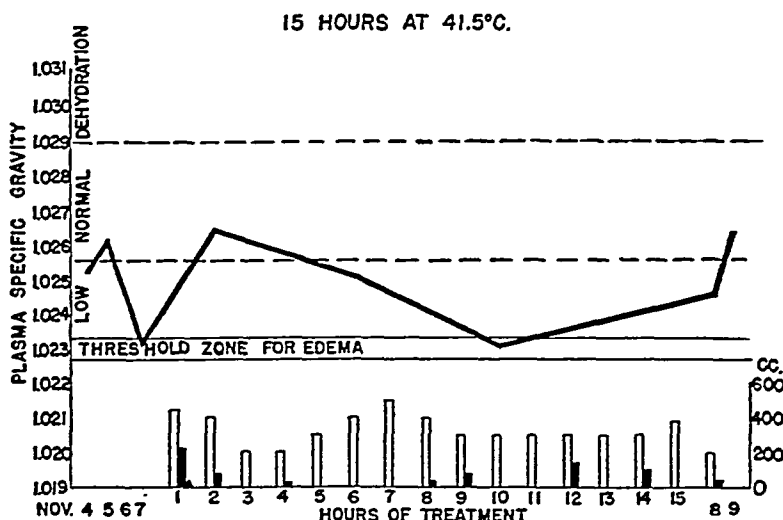


FIG. 8



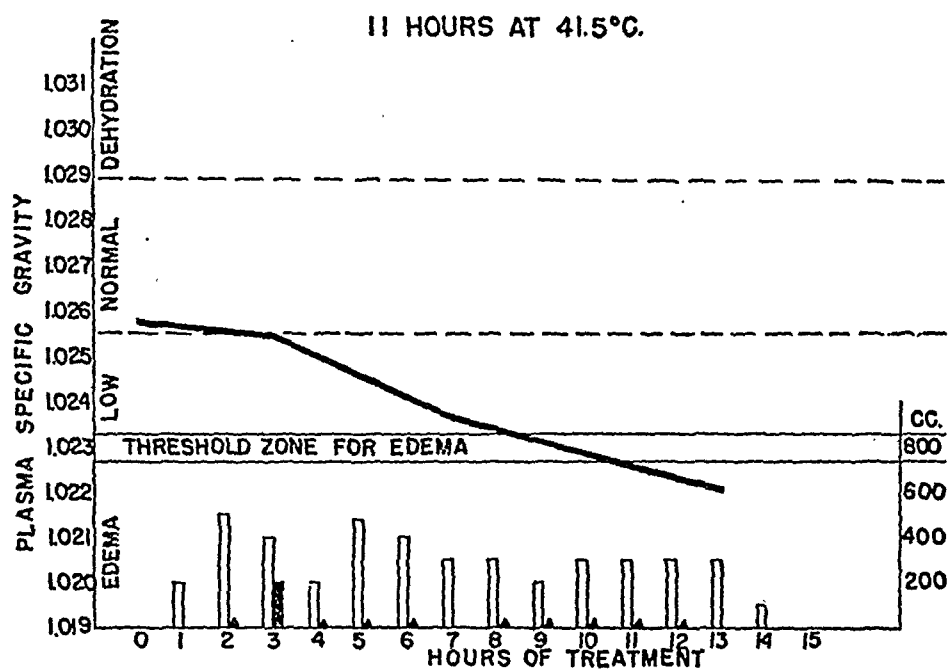


FIG. 9

the two states. The state of overhydration collapse or shock was entirely new to us and we have sometimes been guilty of inducing it in our attempt for optimum adjustment. (7) In this group were 9 definite cases, or 3 per cent of the entire series. Three cases have been chosen to illustrate the changes brought about during the development of a state of overhydration.

Case Number 8 (Figure 8): Patient E. L. developed a state of moderate overhydration. A 23-year-old female with gonorrheal arthritis and cervicitis was treated for 15 hours at 41.5° C. Intake was 5200 cc. of fluid and a total of 12 grams of sodium chloride. The urine output was only 665 cc. Although definitely overhydrated (showing a plasma specific gravity of 1.0232) the patient did not become edematous nor show changes in blood pressure. In general, she tolerated the procedure fairly well and recovered promptly.

Case Number 9 (Figure 9): A 21-year-old female with chorea was given 11 hours of fever at 41.5° C. She received a total of 4300 cc. of fluid and 9 grams of salt over this period of time. Her urinary output was 365 cc., which was unusually scant as compared with the large intake. Sweating was only moderate. Here also, specific gravity readings were not yet used to govern the fluid intake, and from the 7th to the 13th hours, the plasma specific gravity dropped from 1.0237 to 1.0212. In this case, the patient had gross edema, with swelling of the face and eyelids, and pitting edema of the sacrum and legs, indicating that fluids had been forced beyond the body needs. This incident was in accord with the findings of Baird and Haldane (35) who showed that the administration of sodium chloride in excess of the ability to excrete it produced visible edema. Other than the water-

logging, her course was favorable. There was some post-fever nausea and vomiting, and she did seem somewhat more exhausted by the fever treatment than does the average well-controlled case.

Case Number 10: This case developed a severe and almost fatal overhydration, with a clinical syndrome almost indistinguishable from dehydration. A 24-year-old female with advanced congenital paresis, confused, emotionally unstable, agitated, was treated after the usual preparation. She tolerated the treatment at 41.5° C. fairly well up to the 10th hour when she began to have grand mal seizures which passed into status epilepticus, at which time the treatment was terminated. By this time, her intake was 5300 cc. of fluid, urine output was 80 cc. with moderate sweating. She received 0.5 gram of Evipal intravenously and 0.5 gram of Evipal intramuscularly without affecting the attacks. In order to stop the muscular effort from the steady series of seizures which might further elevate the temperature, a total of 65 cc. of venethane ether by inhalation was necessary. Unfortunately, plasma specific gravity measurements were not obtained before treatment, but a reading taken when the Evipal was given was 1.0175, the lowest ever observed. The patient's temperature remained between 40.0° C. and 41.0° C. for the next 12 hours, in spite of efforts to reduce it. The skin was extremely dry, with no evidence of any sweating and there was no urinary output. Because of the extreme overhydration as indicated by the plasma specific gravity, the administration of fluid was withheld. One cc. of salyrgan and 0.4 gram of digitalis were given intravenously for their diuretic effect. Following this, and over the next 12-hour period, the patient excreted 1200 cc. of urine, and by the next afternoon, was on the way to recovery from the excessive fluid storage. The blood pressure during the 10 hours of actual treatment varied between 100/50 and 115/30. This patient was not outwardly

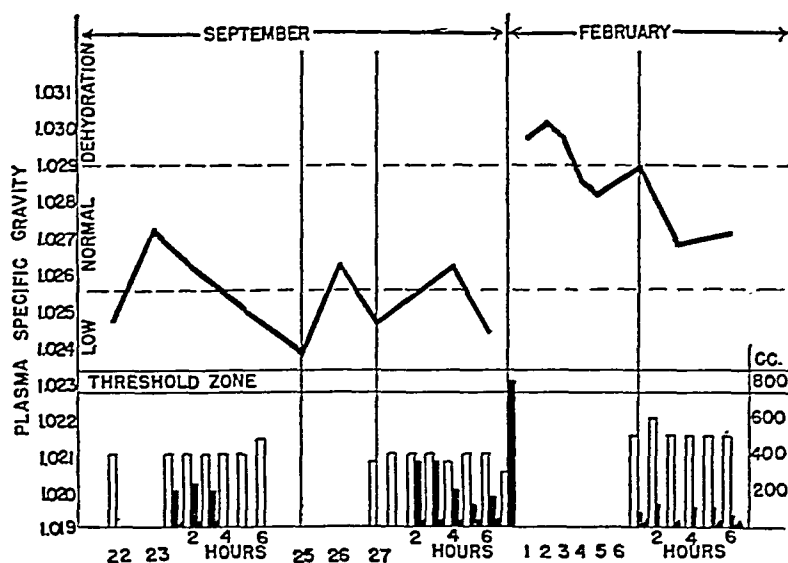
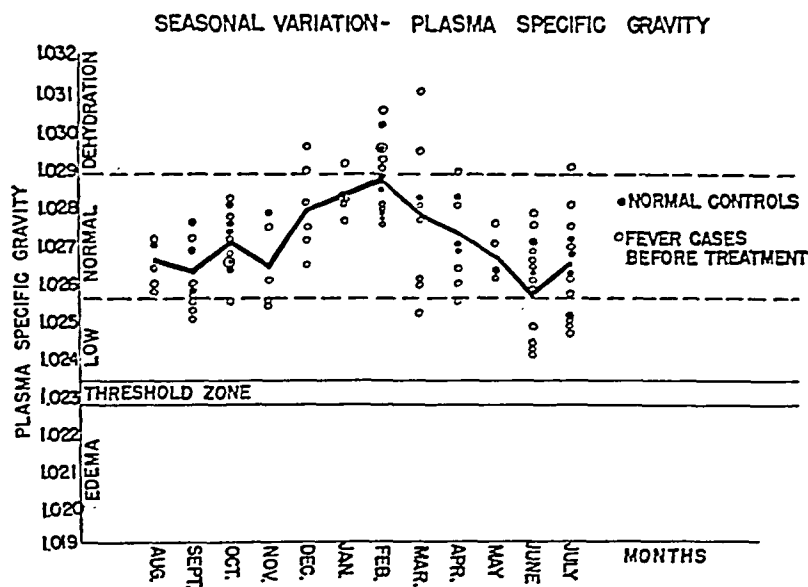
edematous at any time, the overhydration apparently being mostly within the circulating system and brain. This situation may be very confusing to the clinician, for the appearance is all in favor of dehydration. Without the intervention of the anesthetics to stop the seizures, which if they had continued would have raised the body temperature still higher, and the effective application of diuretics to eliminate the excessive fluid, this case would undoubtedly have had a fatal termination.

#### SEASONAL VARIATION

It was noted that during January, February, and March of each year, the plasma specific

gravity values were consistently higher than previously, and that individuals were encountered during these months who could not be "properly" hydrated prior to artificial fever therapy. It was during these months that Cases 7 and 8, discussed under the dehydrated group, were encountered. Representative averages for the last 4 years of normal cases and observations before and after fever treatment have been plotted in Figure 10.

It may be seen that a definite change in the level is established during the late winter months. In



Case Number 11 (Figure 11), observed and treated in September and again in February, there is noted a decided shift of the plasma specific gravity levels. This illustrates an observation noted many times in the same individual receiving fever therapy in two different seasons of the year. Bazett *et al.* (34) has reported differences in blood volume in summer and winter. Barcroft *et al.* (4), by means of the carbon monoxide method of blood volume measurement, showed an average increase of 10 to 25 per cent in blood volume in subjects who travelled from England through the tropics. We conducted several preliminary experiments in which the room temperatures and humidity were elevated to "summer levels" for short periods of 3 to 5 days during the winter. The storage of water was less than 1 per cent and the changes were not in accordance with the magnitudes reported by Bazett (36). The significance of a seasonal variation in hydration is not well understood although it probably is related in some way to the dry cold part of our winter. However, if one is mindful that such a change can take place, certain dangers in fluid management can be eliminated.

#### SUMMARY

The incidence of collapse during artificial fever therapy can be greatly reduced by judicious replacement of water and sodium chloride lost in sweat.

The development of collapse is found to be associated with dehydration and, occasionally, overhydration of the blood plasma.

The symptoms accompanying both of these states resemble those of traumatic shock; pallor, cold extremities, and high body temperature, absence of sweating and urinary excretion, mania or unconsciousness.

There is therefore need for a guide to control the water and salt intake. This is satisfactorily provided by measurement of plasma specific gravity. This easily obtainable datum dependably forecasts the development of collapse and is therefore an extremely important item of procedure in artificial fever therapy.

The authors wish to express their appreciation to Drs. William S. McCann, Samuel Bassett, Henry Keutmann, and Harold C. Hodge for their many suggestions regard-

ing the treatment of our patients; to Dr. Nolan Kaltreider for his blood volume studies during the studies on seasonal variation; to Miss Emmy Lehman, fever nurse during the early years, Mr. Francis W. Bishop, for technical assistance, to two former fever residents, Dr. Gerald O'Hara and Dr. Harold Cohn,—all of whom contributed their part to the successful conduct of these experiments.

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Case Number 11 (Figure 11), observed and treated in September and again in February, there is noted a decided shift of the plasma specific gravity levels. This illustrates an observation noted many times in the same individual receiving fever therapy in two different seasons of the year. Bazett *et al.* (34) has reported differences in blood volume in summer and winter. Barcroft *et al.* (4), by means of the carbon monoxide method of blood volume measurement, showed an average increase of 10 to 25 per cent in blood volume in subjects who travelled from England through the tropics. We conducted several preliminary experiments in which the room temperatures and humidity were elevated to "summer levels" for short periods of 3 to 5 days during the winter. The storage of water was less than 1 per cent and the changes were not in accordance with the magnitudes reported by Bazett (36). The significance of a seasonal variation in hydration is not well understood although it probably is related in some way to the dry cold part of our winter. However, if one is mindful that such a change can take place, certain dangers in fluid management can be eliminated.

#### SUMMARY

The incidence of collapse during artificial fever therapy can be greatly reduced by judicious replacement of water and sodium chloride lost in sweat.

The development of collapse is found to be associated with dehydration and, occasionally, overhydration of the blood plasma.

The symptoms accompanying both of these states resemble those of traumatic shock; pallor, cold extremities, and high body temperature, absence of sweating and urinary excretion, mania or unconsciousness.

There is therefore need for a guide to control the water and salt intake. This is satisfactorily provided by measurement of plasma specific gravity. This easily obtainable datum dependably forecasts the development of collapse and is therefore an extremely important item of procedure in artificial fever therapy.

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ing the treatment of our patients; to Dr. Nolan Kaltreider for his blood volume studies during the studies on seasonal variation; to Miss Emmy Lehman, fever nurse during the early years, Mr. Francis W. Bishop, for technical assistance, to two former fever residents, Dr. Gerald O'Hara and Dr. Harold Cohn,—all of whom contributed their part to the successful conduct of these experiments.

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# FRACTIONATION OF THE SERUM AND PLASMA PROTEINS BY SALT PRECIPITATION IN INFANTS AND CHILDREN.<sup>1</sup> 1. THE CHANGES WITH MATURITY AND AGE. 2. THE CHANGES IN GLOMERULONEPHRITIS. 3. THE CHANGES IN NEPHROSIS

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The various components constituting the plasma protein complex have been characterized by many different chemical methods. Perhaps the most extensively used method of defining the component proteins has been by describing their solubility behavior in salt solutions. Thus, the terms fibrinogen, euglobulin, pseudoglobulin, and albumin have been applied, not to molecular species or chemical entities, but to the fractional parts of the total plasma protein complex, separated by precipitation at specified salt concentrations.

In this study, the plasma protein fractions "salted out" by increasing concentrations of salt solution were quantitated in normal infants and children. The influence of maturity and age upon the concentration of the individual fractions precipitated was studied by examining and comparing the bloods of premature infants, full-term newborn infants, older infants, and children. The quantitative changes occurring during the course of various diseases, especially nephritis and nephrosis, were measured. ✓

## METHODS

The phosphate salt mixture used by Butler *et al.* (1, 2) in their study of the solubility curves of human plasma proteins was employed. These authors, using an equation derived by Cohn (3), obtained a discontinuous curve when the solubility of the protein expressed logarithmically was plotted against increasing concentrations of the phosphate precipitant. They stated that the breaks in the discontinuous curve were caused by successive precipitation of the progressively more soluble protein fractions.

Briefly, the method entailed precipitation of the proteins at room temperature with increasing concentrations of a phosphate solution. The phosphate precipitant was made of equal parts of monobasic and dibasic potassium phosphate, so that the pH of the different concentrations employed was constant at 6.5. Blood was collected without

stasis and allowed to clot. After centrifugation, the serum was separated. One volume of serum was added to 30 volumes of phosphate precipitant. (The different concentrations of phosphate precipitant were prepared from a stock 3 molar solution.) In actual practice, 0.2 cc. of serum was added to 6.0 cc. of phosphate solution; instead of filtrations as practiced by Butler *et al.* (1, 2), the precipitated protein was removed by centrifugation at 2,000 R.P.M. for 15 minutes in the angle centrifuge. Supernatant fluids were analyzed by the micro-Kjeldahl method. The concentrations of phosphate precipitant used with each serum ranged from 0.8 molar to 3.0 molar.

Because there is, as indicated by Butler *et al.* (2), a lack of sharp breaks in the solubility curve of the serum proteins obtained by salt precipitation, we have recorded the absolute amount of protein (grams per 100 cc.) precipitated by each successive increase in salt concentrations of precipitant. In the interest of consistency, we have also designated in our tables the fractions precipitated by the  $\text{Na}_2\text{SO}_4$  method of Howe (4) as the fractions precipitated and soluble in 22 per cent sodium sulphate. The fraction precipitated by this concentration of  $\text{Na}_2\text{SO}_4$  is, in conformity with custom, referred to in the text as total globulin and the protein remaining in solution, total albumin. It is to be emphasized that the protein fractions precipitated by either method employed are not chemical entities and that the terms globulin and albumin are used merely for convenience.

Fibrin was determined by the method of Cullen and Van Slyke (5) on oxalated plasma obtained at the same time as the serum sample.

## I. NORMAL INFANTS AND CHILDREN

Ten healthy children, ranging in age from 5 to 10 years, were studied. Their serums and plasmas were examined on 2 or more occasions. Each determination was done in duplicate. The values for the protein fractions determined by the phosphate precipitant and the Howe sodium sulphate methods were found to be fairly constant for a given child, on determinations done at different times. Table I contains the values for the protein fractions for 2 of the normal children, and is presented as an example of the relatively constant

<sup>1</sup> Aided by a grant from the Mead Johnson Company, Evansville, Indiana.



TABLE I

*Grams of protein per 100 cc. of serum obtained by single fractional precipitation of serum proteins of the normal child on different occasions*

Subject	Date	Fibrin*	1.5 Molar Na <sub>2</sub> SO <sub>4</sub> †		Molar concentrations of phosphate precipitant					
			Insoluble ("Globulin")	Soluble ("Albumin")	1.6 Molar	2.0 Molar	2.4 Molar	2.6 Molar	2.8 Molar	3.0 Molar
D. L. Female 8 years old	May 4, 1939	0.240	1.54	5.14	1.22	1.10	1.30	0.95	1.28	0.80
	May 10, 1939	0.252	1.50	5.07	1.16	0.95	1.06	1.09	1.24	0.80
	May 17, 1939	0.248	1.56	5.15	1.16	1.07	1.29	0.96	1.44	0.60
S. G. Male 6 years old	January 23, 1939	0.347	2.21	4.44	0.95	1.35	0.98	0.83	1.77	0.68
	February 1, 1939	0.334	2.37	4.57	1.02	1.46	0.93	0.83	1.83	0.84

\* Fibrin determined on oxalated plasma by method of Cullen and Van Slyke.

† By method of Howe. The fraction of the serum proteins insoluble in 1.5 Molar Na<sub>2</sub>SO<sub>4</sub> is commonly referred to as globulin, the fraction remaining in solution, as albumin.

values of the protein fractions in the same child. The duplicate determinations done on the same blood sample checked closely. We feel that the validity and reproducibility of our analytical technique were established by these checks.

In Tables II and III are summarized the data obtained from the examination of the serum and plasma proteins of normal infants and children. In these tables and all subsequent tables, the values obtained for the group of 10 children from 5 to 10

years of age are used as the standard of reference. Between these ages, it is known that the plasma proteins have attained adult values (6). Seventeen premature infants, ranging in age from one to 68 days, were studied. All weighed less than 5 pounds at the time of study. Seventeen full-term newborn infants, weighing from 7 to 8½ pounds, were studied during the first 48 hours of life. Sixteen healthy infants, ranging in age from 2 to 11 months, constituted the group of older infants examined. In addition, the serums and plasmas of 6 mothers of the premature infants were analyzed. In the premature and newborn infants and in some of the older infants, blood samples were obtained from the superior longitudinal sinus. When blood was drawn from a vein, it was obtained without stasis.

The concentrations of the protein fractions are expressed as grams per 100 cc. of serum or plasma. The standard deviation of each average value is included. Using the average obtained from the 10 older children as normal standards, the "t" test of Fisher (7) was applied to determine significant difference for the values obtained with the other subjects studied.

Examination of Table II shows that the blood fibrin levels are remarkably constant and similar at all age periods studied. These values are in the normal range for adults (8). The high fibrin values for the mothers of the premature infants is

TABLE II

*Influence of age and maturity on serum proteins in grams per 100 cc.*

	Fibrin	Total serum protein	1.5 Molar Na <sub>2</sub> SO <sub>4</sub>	
			Insoluble†	Soluble†
Normal children (10 children)	0.28±0.04	7.30±0.59	2.4±0.74	5.0±0.78
Premature infants (17 infants) Ages: 1 to 68 days	0.27±0.15	4.55±0.59*	1.01±0.45*	3.55±0.65*
Full-term newborn infants (17 infants)	0.24±0.04	5.11±0.76*	1.34±0.41*	3.76±0.43*
Older infants (16 infants) Ages: 2 to 11 months	0.25±0.03	6.10±0.29*	1.35±0.65*	4.07±0.73
Mothers of premature infants (6 mothers)	0.56±0.05*	7.20±0.59	2.20±0.76	4.83±0.44

\* Significant difference from value for normal children (Fisher's "t" test).

† The fraction in grams per 100 cc. of serum protein precipitated by 1.5 Molar Na<sub>2</sub>SO<sub>4</sub> is commonly referred to as globulin, the amount of serum protein remaining in solution, as albumin.

a characteristic finding in pregnant and postpartum women (9).

The total serum protein concentration rises with increasing maturity, starting with a level of  $4.55 \pm 0.59$  grams per 100 cc. of serum in the premature infant and reaching a value of  $7.30 \pm 0.59$  grams in the normal child. This value for the child from 5 to 10 years is the same as that of the adult. The values for older infants (2 to 11 months) are still significantly below the adult figure. We have no data covering the age period from 1 to 5 years, so that we cannot state at which time the normal adult level of total serum protein is attained.

As is obvious in Table II, the increase of total serum protein concentration with age is dependent upon increases in the 2 fractions commonly designated as globulin and albumin. However, in the attainment of the adult level of total serum protein, there is a proportionately greater increase in globulin concentration than in albumin. The low value of the globulin fraction in the premature infant ( $1.01 \pm 0.45$  grams) is in good agreement with the values obtained by Darrow and Cary (10). It is interesting to note that the albumin concentration reaches the adult level in the infant at a time when the globulin concentration is still low. Achard and his co-workers (11) reported that the serum globulin of the newborn infant was lower than that of the maternal serum, but that the serum albumin was of the same order of magnitude in both infant and mother. However, our data indicate that the serum albumin of pre-

mature and full-term newborn infants is significantly lower than the maternal serum albumin.

Table III summarizes the data obtained by fractionation of the serums of the same individuals represented in Table II, with the phosphate precipitant. We have charted our data as increments of protein (in grams per 100 cc. of serum) precipitated by successive increases of 0.4 molarity of phosphate precipitant from 0.8 to 2.4 molarity. From 2.4 to 3.0 molar concentration, the protein fractions were separated by successive increases of 0.2 molarity.

Because of technical difficulty in obtaining clear supernatant fluids after centrifugation of the protein precipitates with the 0.8 and 1.2 molar phosphate solutions, we do not feel justified in drawing any conclusions concerning any existing differences in the amounts of protein salted out by these concentrations of precipitant. From 1.2 to 3.0 molar concentration of phosphate precipitant, the supernatant fluids were always clear and subject to accurate analysis. It appears that some of the fraction termed albumin by Howe is present in the precipitate below 2.0 molar phosphate solution, since the summation of the protein increments precipitated below this level is greater than the total globulin value obtained by the  $\text{Na}_2\text{SO}_4$  method. Also, from the nature of the precipitation curves (1), it would appear that some of the fraction termed globulin may be in solution above 2.0 molar concentration. Thus it may be repeated that, in reporting these increments of protein precipitated by the phosphate or  $\text{Na}_2\text{SO}_4$  precipitants,

TABLE III  
*Effect of age and maturity on precipitation pattern of serum proteins*

Molar concentration of phosphate precipitant	Grams of protein precipitated per 100 cc.							
	0.8	1.2	1.6	2.0	2.4	2.6	2.8	3.0
Normal children (10 children)	$0.12 \pm 0.06$	$0.30 \pm 0.13$	$1.11 \pm 0.22$	$1.21 \pm 0.19$	$1.16 \pm 0.25$	$0.88 \pm 0.17$	$1.68 \pm 0.43$	$0.71 \pm 0.28$
Premature infants (17 infants) Ages: 1 to 68 days	$0.05 \pm 0.07$	$0.19 \pm 0.15$	$0.53 \pm 0.23^*$	$0.85 \pm 0.09^*$	$0.83 \pm 0.16^*$	$0.59 \pm 0.13^*$	$0.89 \pm 0.17^*$	$0.49 \pm 0.18$
Full-term newborn infants (17 infants)	$0.06 \pm 0.12$	$0.20 \pm 0.10$	$0.68 \pm 0.17^*$	$0.99 \pm 0.17^*$	$0.99 \pm 0.19$	$0.66 \pm 0.12^*$	$0.87 \pm 0.24^*$	$0.50 \pm 0.18$
Older infants (16 infants) Ages: 2 to 11 mos.	$0.02 \pm 0.08^*$	$0.06 \pm 0.07^*$	$0.52 \pm 0.09^*$	$1.18 \pm 0.16$	$1.46 \pm 0.20^*$	$1.02 \pm 0.16^*$	$1.29 \pm 0.24$	$0.32 \pm 0.12^*$
Mothers of premature infants (6 mothers)	$0.13 \pm 0.12$	$0.26 \pm 0.08$	$1.03 \pm 0.17$	$1.28 \pm 0.30$	$1.15 \pm 0.10$	$0.94 \pm 0.17$	$1.23 \pm 0.18$	$0.45 \pm 0.41$

\* Significant difference from value for normal children (Fisher's "t" test).

we are not implying chemical specificity for the fractions. However, we feel that the fractions in a sense may be designated as entities for purposes of comparison because of their reproducibility.

In the present state of our knowledge, there appear to be at least 4 electrochemical protein components in the serum protein complex, *viz.*, gamma, beta, and alpha globulin and albumin, named in order of increasing mobility in the electrophoretic cell (12, 13). While the globulin fractions obtained by the Howe method have been correlated with the globulins identified by electrophoretic analysis (14), we have no data comparing the fractions obtained by the phosphate precipitation to electrophoretic fractions. However, there is evidence to suggest that the protein with the lowest mobility (gamma globulin) is salted out by the lowest concentration of precipitant, and that with increasing concentration of precipitant, the proteins of increasing electrophoretic mobility are salted out progressively (14, 15, 16).

From Table III, it is apparent that the values for each of the protein fractions precipitated by concentrations of phosphate precipitant from 1.2 to 2.8 molar are significantly low in the premature infant. Similar low values are encountered in the full-term newborn infant, except that the protein fraction precipitating between 2.0 and 2.4 molar concentration is not significantly low. In the older infant, the low values are only in the range below 1.6 molar concentration. At the higher concentrations of phosphate precipitant, from 2.0 to 3.0 molar concentration, the changes in the amounts of protein precipitated exhibit no constant trend, 2 of the fractions being high and 2, low in value. The reason for these variations is not apparent, but it is possible that the varying proportions of albumin to globulin with increasing age may in some way be responsible for these alterations in precipitation pattern.

Even though there is a progressive increase in total globulin with increasing maturity (Tables II and III), it is apparent that the protein fraction precipitated below 1.6 molar phosphate is lowered throughout the entire period of infancy, having the same value in the older infant as in the premature. This fraction has been said to be chiefly the euglobulin fraction (1, 2). The rise in total globulin occurring with increasing maturity of the

infant is reflected, at least in part, in the progressive increase in value of the fraction precipitated between 1.6 and 2.0 molar phosphate. Since it seems that the globulins with the lowest electrophoretic mobility are salted out first, it is likely that the serum protein fraction precipitated below 1.6 molarity is chiefly gamma globulin, the fraction which has been shown to contain many immune bodies. One is tempted to correlate the low concentration of this fraction in the infant with his well-known poor immune responses (17).

There is no apparent difference in the protein fractions of the older normal child and the adult woman, as is obvious from the values obtained for the 6 mothers of premature infants.

## II. CHILDREN WITH GLOMERULONEPHRITIS

The plasmas and serums of a group of children in different stages of glomerulonephritis were examined and the results are summarized in Tables IV and V. In Table IV, the values for the pro-

TABLE IV  
*Serum proteins in nephritis as grams per 100 cc.*

	Fibrin	Total serum protein	1.5 Molar $\text{Na}_2\text{SO}_4$ precipitant	
			Insoluble†	Soluble†
Normal children (10 children)	$0.28 \pm 0.04$	$7.30 \pm 0.59$	$2.4 \pm 0.74$	$5.0 \pm 0.78$
Nephritis: active stage (20 children)	$0.42 \pm 0.11^*$	$7.04 \pm 0.85$	$2.8 \pm 0.95$	$4.24 \pm 0.97$
Nephritis: chronic stage (4 children)	$0.43 \pm 0.20^*$	$5.03 \pm 0.94$	$1.28 \pm 0.20^*$	$3.75 \pm 0.83^*$
Nephritis: healed stage (7 children)	$0.24 \pm 0.06$	$7.11 \pm 0.25$	$1.73 \pm 0.42$	$5.39 \pm 0.38$

\* Significant difference from value for normal children (Fisher's "t" test).

† The fraction in grams per 100 cc. of serum protein precipitated by 1.5 Molar  $\text{Na}_2\text{SO}_4$  is commonly referred to as globulin, the amount of serum protein remaining in solution, as albumin.

tein fractions usually termed fibrin, albumin, and globulin are tabulated. The normal reference values are again the group of 10 healthy children, aged 6 to 10 years. In the active stage of acute glomerulonephritis, there is an increase in blood fibrin which returns to normal with healing. In patients in the chronic stage of glomerulonephritis, this elevation of fibrin persists. The total serum protein is reduced during the active stage of acute

TABLE V  
Precipitation pattern of serum proteins in nephritis

Molar concentration of phosphate precipitant	Grams of protein precipitated per 100 cc.							
	0.8	1.2	1.6	2.0	2.4	2.6	2.8	3.0
Normal children (10 children)	0.12 ± 0.06	0.30 ± 0.13	1.11 ± 0.22	1.21 ± 0.19	1.16 ± 0.25	0.88 ± 0.17	1.68 ± 0.43	0.71 ± 0.28
Nephritis: active stage (20 children)	0.12 ± 0.12	0.29 ± 0.19	1.57 ± 0.42*	1.38 ± 0.30	1.25 ± 0.36	0.90 ± 0.32	0.98 ± 0.30*	0.45 ± 0.21*
Nephritis: chronic stage (4 children)	0.04 ± 0.09	0.11 ± 0.09*	0.65 ± 0.16*	1.35 ± 0.34	0.94 ± 0.28	0.74 ± 0.27	0.82 ± 0.23*	0.32 ± 0.21*
Nephritis: healed stage (7 children)	0.03 ± 0.07	0.20 ± 0.07	1.02 ± 0.17	1.23 ± 0.15	1.43 ± 0.15	1.48 ± 0.29		0.82 ± 0.05

\* Significant difference from value for normal children (Fisher's "t" test).

glomerulonephritis, the reduction being caused by a lowering of the sodium sulphate albumin fraction. These reductions were not statistically significant. In the chronic stage of glomerulonephritis, there is a persistently significant reduction in both the sodium sulphate globulin and albumin fractions.

Table V summarizes the values obtained with the phosphate precipitant. The protein fraction precipitating below 1.6 molar phosphate is significantly increased in amount in patients in the acute stage of glomerulonephritis. This significant increase in a sub-fraction of the total globulin (probably gamma globulin) is obscured on determination of the total globulin by the Howe method (Table IV). With healing, this fraction assumes its normal value. In contrast to the acute nephritic, the patients in the chronic stage of glomerulonephritis have a reduction in this fraction. This is in keeping with the observation of Kendall (18) who has demonstrated, by an immunological method, a decrease in a globulin fraction in chronic nephritis. The decrease in the protein fractions precipitated from 2.6 to 3.0 molar concentration, in both the acute and chronic nephritic, corresponds to a decrease in what Butler *et al.* term Albumin II (2). The finding of this decrease is especially interesting in the active stage of acute nephritis where it is not apparent when total albumin is determined alone. In the patients with chronic nephritis, the reduction in the fractions precipitated between 2.6 and 3.0 molar concentration accounts for the total reduction in the albumin fraction as determined by the Howe method.

As criteria of healing in glomerulonephritis, we have utilized the blood sedimentation rate and the Addis count. In another publication (19), we have pointed out that the blood sedimentation rate is usually rapid as long as activity of the nephritis continues, and becomes normal as the process heals. Ham and Curtis (20) have pointed out a direct correlation between the rapidity of blood sedimentation and the plasma fibrin level. Our data would seem to confirm this observation. In the patient with acute glomerulonephritis, the plasma fibrin is increased, as is the sedimentation rate, both returning to normal with healing. Furthermore, in chronic nephritis, there is a sustained increase in both plasma fibrin and blood sedimentation rate. This correlation is pointed out with the usual reservation that a positive correlation does not necessarily imply cause and effect. It has been stated also that an elevation of the serum globulin was correlated with a rapid blood sedimentation rate (20). This is not in keeping with our data. Since, while the patient with active acute glomerulonephritis had an elevated globulin at a time when his blood sedimentation was rapid, the chronic nephritic showed the reverse relationship, *viz.*, a lowered total globulin and a rapid blood sedimentation rate.

We have already suggested that the protein fraction precipitated below 1.6 molar phosphate is probably largely so-called gamma globulin and a carrier of antibodies. The increase in amount of this fraction in the acute stage of glomerulonephritis may be correlated with an increase in the antistreptolysin titer found during the acute phase of this disease (21), and the return of this frac-

we are not implying chemical specificity for the fractions. However, we feel that the fractions in a sense may be designated as entities for purposes of comparison because of their reproducibility.

In the present state of our knowledge, there appear to be at least 4 electrochemical protein components in the serum protein complex, *viz.*, gamma, beta, and alpha globulin and albumin, named in order of increasing mobility in the electrophoretic cell (12, 13). While the globulin fractions obtained by the Howe method have been correlated with the globulins identified by electrophoretic analysis (14), we have no data comparing the fractions obtained by the phosphate precipitation to electrophoretic fractions. However, there is evidence to suggest that the protein with the lowest mobility (gamma globulin) is salted out by the lowest concentration of precipitant, and that with increasing concentration of precipitant, the proteins of increasing electrophoretic mobility are salted out progressively (14, 15, 16).

From Table III, it is apparent that the values for each of the protein fractions precipitated by concentrations of phosphate precipitant from 1.2 to 2.8 molar are significantly low in the premature infant. Similar low values are encountered in the full-term newborn infant, except that the protein fraction precipitating between 2.0 and 2.4 molar concentration is not significantly low. In the older infant, the low values are only in the range below 1.6 molar concentration. At the higher concentrations of phosphate precipitant, from 2.0 to 3.0 molar concentration, the changes in the amounts of protein precipitated exhibit no constant trend, 2 of the fractions being high and 2, low in value. The reason for these variations is not apparent, but it is possible that the varying proportions of albumin to globulin with increasing age may in some way be responsible for these alterations in precipitation pattern.

Even though there is a progressive increase in total globulin with increasing maturity (Tables II and III), it is apparent that the protein fraction precipitated below 1.6 molar phosphate is lowered throughout the entire period of infancy, having the same value in the older infant as in the premature. This fraction has been said to be chiefly the euglobulin fraction (1, 2). The rise in total globulin occurring with increasing maturity of the

infant is reflected, at least in part, in the progressive increase in value of the fraction precipitated between 1.6 and 2.0 molar phosphate. Since it seems that the globulins with the lowest electrophoretic mobility are salted out first, it is likely that the serum protein fraction precipitated below 1.6 molarity is chiefly gamma globulin, the fraction which has been shown to contain many immune bodies. One is tempted to correlate the low concentration of this fraction in the infant with his well-known poor immune responses (17).

There is no apparent difference in the protein fractions of the older normal child and the adult woman, as is obvious from the values obtained for the 6 mothers of premature infants.

## II. CHILDREN WITH GLOMERULONEPHRITIS

The plasmas and serums of a group of children in different stages of glomerulonephritis were examined and the results are summarized in Tables IV and V. In Table IV, the values for the pro-

TABLE IV  
*Serum proteins in nephritis as grams per 100 cc.*

	Fibrin	Total serum protein	1.5 Molar Na <sub>2</sub> SO <sub>4</sub> precipitant	
			Insoluble†	Soluble†
Normal children (10 children)	0.28±0.04	7.30±0.59	2.4±0.74	5.0±0.78
Nephritis: active stage (20 children)	0.42±0.11*	7.04±0.85	2.8±0.95	4.24±0.97
Nephritis: chronic stage (4 children)	0.43±0.20*	5.03±0.94	1.28±0.20*	3.75±0.83*
Nephritis: healed stage (7 children)	0.24±0.06	7.11±0.25	1.73±0.42	5.39±0.38

\* Significant difference from value for normal children (Fisher's "t" test).

† The fraction in grams per 100 cc. of serum protein precipitated by 1.5 Molar Na<sub>2</sub>SO<sub>4</sub> is commonly referred to as globulin, the amount of serum protein remaining in solution, as albumin.

tein fractions usually termed fibrin, albumin, and globulin are tabulated. The normal reference values are again the group of 10 healthy children, aged 6 to 10 years. In the active stage of acute glomerulonephritis, there is an increase in blood fibrin which returns to normal with healing. In patients in the chronic stage of glomerulonephritis, this elevation of fibrin persists. The total serum protein is reduced during the active stage of acute

TABLE V  
Precipitation pattern of serum proteins in nephritis

Molar concentration of phosphate precipitant	Grams of protein precipitated per 100 cc.							
	0.8	1.2	1.6	2.0	2.4	2.6	2.8	3.0
Normal children (10 children)	0.12 ± 0.06	0.30 ± 0.13	1.11 ± 0.22	1.21 ± 0.19	1.16 ± 0.25	0.88 ± 0.17	1.68 ± 0.43	0.71 ± 0.28
Nephritis: active stage (20 children)	0.12 ± 0.12	0.29 ± 0.19	1.57 ± 0.42*	1.38 ± 0.30	1.25 ± 0.36	0.90 ± 0.32	0.98 ± 0.30*	0.45 ± 0.21*
Nephritis: chronic stage (4 children)	0.04 ± 0.09	0.11 ± 0.09*	0.65 ± 0.16*	1.35 ± 0.34	0.94 ± 0.28	0.74 ± 0.27	0.82 ± 0.23*	0.32 ± 0.21*
Nephritis: healed stage (7 children)	0.03 ± 0.07	0.20 ± 0.07	1.02 ± 0.17	1.23 ± 0.15	1.43 ± 0.15	1.48 ± 0.29		0.82 ± 0.05

\* Significant difference from value for normal children (Fisher's "t" test).

glomerulonephritis, the reduction being caused by a lowering of the sodium sulphate albumin fraction. These reductions were not statistically significant. In the chronic stage of glomerulonephritis, there is a persistently significant reduction in both the sodium sulphate globulin and albumin fractions.

Table V summarizes the values obtained with the phosphate precipitant. The protein fraction precipitating below 1.6 molar phosphate is significantly increased in amount in patients in the acute stage of glomerulonephritis. This significant increase in a sub-fraction of the total globulin (probably gamma globulin) is obscured on determination of the total globulin by the Howe method (Table IV). With healing, this fraction assumes its normal value. In contrast to the acute nephritic, the patients in the chronic stage of glomerulonephritis have a reduction in this fraction. This is in keeping with the observation of Kendall (18) who has demonstrated, by an immunological method, a decrease in a globulin fraction in chronic nephritis. The decrease in the protein fractions precipitated from 2.6 to 3.0 molar concentration, in both the acute and chronic nephritic, corresponds to a decrease in what Butler *et al.* term Albumin II (2). The finding of this decrease is especially interesting in the active stage of acute nephritis where it is not apparent when total albumin is determined alone. In the patients with chronic nephritis, the reduction in the fractions precipitated between 2.6 and 3.0 molar concentration accounts for the total reduction in the albumin fraction as determined by the Howe method.

As criteria of healing in glomerulonephritis, we have utilized the blood sedimentation rate and the Addis count. In another publication (19), we have pointed out that the blood sedimentation rate is usually rapid as long as activity of the nephritis continues, and becomes normal as the process heals. Ham and Curtis (20) have pointed out a direct correlation between the rapidity of blood sedimentation and the plasma fibrin level. Our data would seem to confirm this observation. In the patient with acute glomerulonephritis, the plasma fibrin is increased, as is the sedimentation rate, both returning to normal with healing. Furthermore, in chronic nephritis, there is a sustained increase in both plasma fibrin and blood sedimentation rate. This correlation is pointed out with the usual reservation that a positive correlation does not necessarily imply cause and effect. It has been stated also that an elevation of the serum globulin was correlated with a rapid blood sedimentation rate (20). This is not in keeping with our data. Since, while the patient with active acute glomerulonephritis had an elevated globulin at a time when his blood sedimentation was rapid, the chronic nephritic showed the reverse relationship, *viz.*, a lowered total globulin and a rapid blood sedimentation rate.

We have already suggested that the protein fraction precipitated below 1.6 molar phosphate is probably largely so-called gamma globulin and a carrier of antibodies. The increase in amount of this fraction in the acute stage of glomerulonephritis may be correlated with an increase in the antistreptolysin titer found during the acute phase of this disease (21), and the return of this frac-

tion to a normal level with healing of the disease coincides with the fall in antistreptolysin titer occurring during recovery. It is also pertinent that the chronic nephritic, in the absence of active infection, exhibits a decrease in this fraction and does not have a very high antistreptolysin titer (22).

### III. CHILDREN WITH NEPHROSIS

The plasmas and serums of 11 children with the nephrotic syndrome were examined 2 or more times during periods when they were in an active phase of their disease, as evidenced by edema,

TABLE VI

*Serum proteins in nephrosis as grams per 100 cc.*

	Fibrin	Total serum protein	1.5 Molar $\text{Na}_2\text{SO}_4$ precipitant	
			Insoluble†	Soluble†
Normal children (10 children)	$0.28 \pm 0.04$	$7.30 \pm 0.59$	$2.40 \pm 0.74$	$5.0 \pm 0.78$
Nephrosis: active stage (11 children) Observed in 26 active periods	$0.65 \pm 0.17^*$	$3.88 \pm 0.77^*$	$2.70 \pm 0.70$	$1.19 \pm 0.88^*$
Nephrosis: inactive stage (6 children)	$0.67 \pm 0.30^*$	$5.89 \pm 1.30^*$	$2.23 \pm 0.29$	$3.52 \pm 1.54^*$

\* Significant difference from value for normal children (Fisher's "t" test).

† The fraction in grams per 100 cc. of serum protein precipitated by 1.5 Molar  $\text{Na}_2\text{SO}_4$  is commonly referred to as globulin, the amount of serum protein remaining in solution, as albumin.

massive albuminuria, and hypercholesterolemia. Fractionation by the Howe technic (Table VI) shows the well-known changes of this disease: normal or slightly elevated sodium sulphate globulin values, low sodium sulphate albumin values.

Six of these children were re-examined during an inactive phase, when they had been free from edema and apparently "clinically well" for a 3-month period. It is apparent that the fibrinogen value was still elevated, perhaps related to the still rapid sedimentation rate. Similarly, evidence of incomplete recovery was also apparent from the reduced serum albumin.

In the fractions obtained by the phosphate precipitation (Table VII), the striking finding is the reduction in the globulin fraction precipitated between 1.2 and 1.6 molar concentrations, which became normal when the disease was apparently inactive clinically (Table VIII).

This reduction is similar to the lowered value found in chronic nephritis. However, there is this difference; in the chronic glomerulonephritic, the total globulin value by  $\text{Na}_2\text{SO}_4$  precipitation is low, while in the nephrotic the total globulin value by the same method is normal. One may thus interpret this finding as an indication that in both diseases there is a change in a specific globulin moiety (gamma globulin).

Since the total sodium sulphate globulin value for the active nephrotic is normal or slightly elevated (2.7 grams per 100 cc. of serum) and since all the proteins, precipitating from 1.6 molar phosphate concentration and below, total only 0.87 grams, a large part of the globulin (1.83 grams) appears to reside in the fraction precipitating between 1.6 and 2.0 molarity and in fractions above this concentration of phosphate precipitant.

This apparent altered dispersion of the globulin fractions, obtained by phosphate precipitation in the nephrotic patient, was subjected to further study. Because of the well-known elevated lipid

TABLE VII

*Precipitation pattern of serum proteins in nephrosis*

Molar concentration of phosphate precipitant	Grams of protein precipitated per 100 cc.							
	0.8	1.2	1.6	2.0	2.4	2.6	2.8	3.0
Normal children (10 children)	$0.12 \pm 0.06$	$0.30 \pm 0.13$	$1.11 \pm 0.22$	$1.21 \pm 0.19$	$1.16 \pm 0.25$	$0.88 \pm 0.17$	$1.68 \pm 0.43$	$0.71 \pm 0.28$
Nephrosis: active stage (11 children) 26 periods of observation	$0.23 \pm 0.16$	$0.20 \pm 0.14$	$0.44 \pm 0.20^*$	$1.43 \pm 0.46$	$1.00 \pm 0.40$	$0.23 \pm 0.13^*$	$0.15 \pm 0.17^*$	$0.03 \pm 0.01^*$
Nephrosis: inactive stage (6 children)	$0.23 \pm 0.22$	$0.19 \pm 0.06$	$1.25 \pm 0.23$	$1.50 \pm 0.19^*$	$1.04 \pm 0.07$	$0.62 \pm 0.33$	$0.65 \pm 0.47^*$	$0.42 \pm 0.14$

\* Significant difference from value for normal children (Fisher's "t" test).

TABLE VIII

*Changes in serum and plasma protein fractions, in grams per 100 cc., of a patient during subsidence of nephrotic syndrome*

Date	Clinical condition of patient	Fibrin	Total serum protein	1.5 Molar Na <sub>2</sub> SO <sub>4</sub> precipitant		Precipitated by increasing concentrations of phosphate precipitant						
				In-soluble	Soluble	0 to 1.2 Molar	1.2 to 1.6	1.6 to 2.0	2.0 to 2.4	2.4 to 2.6	2.6 to 2.8	2.8 to 3.0
February 3, 1939	Edematous (clinically active)	0.87	4.28	3.91	0.37	0.17	0.36	2.05	0.98	0.58		
February 6, 1939	Edematous (clinically active)	0.78	4.47	4.08	0.39	0.46	0.38	2.05	1.42			
February 21, 1939	Edema almost gone	0.55	5.70	4.57	1.13	0.54	0.89	2.17	1.14	0.92		
March 27, 1939	No edema (clinically inactive)	0.42	6.65	2.19	4.46	0.56	1.08	1.56	1.08	0.74	1.11	0.44
April 12, 1939	No edema (clinically inactive)	0.38	7.16	1.92	5.24	0.37	1.47	1.46	0.97	0.91	1.11	0.64

content of the plasma of nephrotic individuals, the effect of removal of serum and plasma fat on the globulin dispersion pattern was investigated.

Samples of plasma and serum of 7 nephrotic patients were obtained during an active stage of the disease and divided into 2 aliquots. One serum and plasma aliquot was subjected to the analytic procedures described before. The second aliquot was shaken with 2 volumes of ether, allowed to stand, and the supernatant ether transferred to weighing bottles. This extraction was done 7 times with each sample. After the last extraction, the residual ether was allowed to evaporate at room temperature, under a gentle stream of air. The residual serum sample was thus brought back to its original volume.<sup>2</sup> Following the removal of fat, the serum was subjected to the same analytical procedures as its non-ether extracted aliquot.

Table IX contains the data showing the effect of the removal of fat on the protein dispersion. It will be noted that in all but one instance, the values for the protein fractions precipitating at 1.6 molar concentration and below are increased significantly, following the removal of fat. Con-

versely, the fractions precipitating at 2.0 molar concentration were usually reduced.

In order further to determine whether hyperlipemia was responsible for the peculiarities of precipitation in the nephrotic patient, the sera of premature infants, who also have a low 1.6 protein fraction, were similarly studied. Ether extraction of 3 serum samples resulted in no significant alteration. It is pertinent that the amount of fat obtained by ether extraction of the premature infant's serum was much lower than that of the nephrotic child's. The fat removed from the premature infant's serum varied from 0.1 to 0.21 gram per 100 cc., while the nephrotic serum samples yielded from 0.77 to 2.04 grams. The hyperlipemic plasma of a child with cretinism yielding 0.612 grams of fat per 100 cc. with ether extraction was also studied. Following ether extraction, the protein precipitation pattern changed in the same directions as that of the nephrotic patient, the 1.6 protein fraction increasing significantly. Fat extraction of a recovered nephritic patient's serum produced no change in the concentration of the 1.6 fraction. This patient's serum yielded a relatively small amount of fat (0.23 gram). Similar treatment of a sample of pooled adult serum resulted in no change in precipitation, again with a small fat yield.

From these considerations, it appears that an increased amount of plasma or serum fat alters the precipitation pattern of the proteins with the phosphate precipitant. Thus, hyperlipemia in the

<sup>2</sup> There is little difficulty in the ether extraction of fat from the serum of normal children or premature infants. However, fatty nephrotic sera, when ether extracted, are likely to form an emulsion of much greater volume than the original serum. These emulsions may be resolved by partial evaporation of the ether and then allowing the sample to stand in the ice-box overnight. The residual supernatant ether which separates out is very fatty.



TABLE IX

*Effect of removal of fat by cold ether extraction on precipitation of protein fractions (grams per 100 cc.) of nephrotic plasmas and serums*

Subject	Condition of serum and plasma	Grams per 100 cc. of serum protein precipitated by phosphate precipitant of increasing molar strength						Fibrin	Total serum protein	1.5 Molar Na <sub>2</sub> SO <sub>4</sub> precipitant	
		1.6 Molar	2.0 Molar	2.4 Molar	2.6 Molar	2.8 Molar	3.0 Molar			In-soluble†	Soluble†
N. B.	Untreated	0.61	1.66	1.40	0.34	0.00	0.01	0.74	4.53	2.19	2.44
	Ether extracted	1.41	1.16	1.14	0.15	0.39		0.74	4.74*	2.76	1.98
N. B.	Untreated	0.64	1.79	1.21	0.57	0.72		0.50	5.49	2.09	3.40
	Ether extracted	1.41	1.28	1.16	0.71	0.81		0.50	6.30*	2.74	3.56
D. B.	Untreated	0.33	0.94	0.54	0.11	0.05			2.31	1.90	0.41
	Ether extracted	0.45	0.92	0.46	0.12	0.00			2.29	1.91	0.37
J. B.	Untreated	0.81	1.42					0.65	3.53	2.65	0.88
	Ether extracted	1.33	1.13					0.65	3.57	2.72	0.85
S. K.	Untreated	0.78	1.77	0.87	0.06	0.07	0.00	0.75	3.59	3.08	0.51
	Ether extracted	1.26	1.31	0.80	0.14	0.06		0.75	3.60	2.93	0.67
L. W.	Untreated	0.61	1.77					0.74	4.68	2.20	2.47
	Ether extracted	2.82						0.74	4.72		
V. D.	Untreated	0.66	1.58					0.48	3.95	2.08	1.87
	Ether extracted	0.99						0.48	3.80		

† The fraction in grams per 100 cc. of serum protein precipitated by 1.5 Molar Na<sub>2</sub>SO<sub>4</sub> is commonly referred to as globulin, the amount of serum protein remaining in solution, as albumin.

\* The apparent increase in total serum protein concentration following ether extraction of these 2 serums was due to evaporation of water from the serums during the evaporation of ether. However, these changes do not alter the significance of the changes in the fractions precipitated by 1.6 and 2.0 molar phosphate precipitant.

nephrotic appears to have some causal relationship to the low protein values at 1.6 (and lower) molar concentrations of precipitant. An increase in serum fat apparently changes the dispersion of globulins, causing them to be precipitated in the range in which the albumins begin to precipitate.

An experiment performed on a normal 8-year-old girl would seem to lend support to this statement. The serum of this child was fractionated following an 18-hour fast. As is apparent from Table X, ether extraction of this serum yielded 230 mgm. of fat per 100 cc. of serum and resulted in no apparent change in the 1.6 and 2.0 fractions. A serum sample taken from the same child, 2 hours after a high fat meal, yielded 530 mgm. of fat on extraction with ether. Following ether extraction, there were shifts in the 1.6 and 2.0 fractions, similar to those encountered in the nephrotic patient.

Obviously, the low 1.6 protein fraction in the premature infant, the full-term newborn infant, and the normal older infant are not dependent

TABLE X

*Effect of increased blood fat on the precipitation pattern of serum proteins with phosphate precipitant*

		Amount of fat removed by ether extraction	Grams per 100 cc. of protein precipitated by phosphate precipitant	
		mgm. per 100 cc.	1.2 to 1.6 Molar	1.6 to 2.0 Molar
C. L. Serum from normal patient after 18 hour fast	Untreated serum		1.05	1.05
	Same serum extracted with cold ether	230	1.02	0.95
C. L. Serum from same patient 2 hours after high fat meal	Untreated serum		0.94	1.22
	Same serum extracted with cold ether	530	1.32	1.03

upon this mechanism, but represent real low values, which reflect the low value for total globulin in these subjects.

Analysis of nephrotic serum by electrophoresis by Longsworth and MacInnes (23), and Luetscher (24) showed a reduction in gamma globulin and high values for alpha and beta globulins. Our analytical data with phosphate precipitation of nephrotic sera would seem to fit in with these findings, in that the 1.6 fraction is below the normal value and the 2.0 fraction tends to be above normal. Following extraction of serum fat with ether, Longsworth and MacInnes found a reduction in both alpha and beta globulin fractions, with no change in concentration of gamma globulin. This is not in keeping with our findings, in that an increase in the 1.6 fraction (gamma globulin) accompanied the decrease in the 2.0 fraction after removal of serum fat with ether. Longsworth and MacInnes regard the decrease in alpha and beta globulins as being due to refractometric changes resulting from the removal of fat with a refractive index similar to that of the serum globulins. Obviously, this explanation does not hold for our data, in that actual changes in nitrogen content of the fractions precipitated were measured. We are inclined to believe that an altered distribution of globulins occurs in the nephrotic patient.

Longcope (21) has noted that the antistreptolysin titer of nephrotic patients was usually low; Earle and his co-workers (22) have observed that the basal antistreptolysin titer of the patient with chronic nephritis falls to a lower level with the onset of a nephrotic stage, returning to a normal titer with the subsidence of edema. It is possible that the elevated blood fat of the nephrotic, through an altered distribution of globulins, may have some bearing on these lowered antistreptolysin values.

While all the protein fractions at the albumin end of the phosphate precipitation have lowered values in the nephrotic patient during the active stage of his disease, the reduction is more marked in the protein fractions precipitating between 2.6 and 3.0 molar concentration of precipitant, the so-called Albumin II. In the inactive stage of the disease, the values for the albumin fractions tend to return toward normal levels. It is apparent that these inactive patients are still abnormal,

since the total albumin as measured by the Howe method was still reduced. Our data suggest the possibility of the existence of a labile albumin component, which fluctuates in concentration with the clinical state of the nephrotic patient, being low during the edematous stages and rising with the disappearance of edema (Table VIII). A similar change of lesser degree is to be noted in the patient with acute nephritis. There is evidence available to show that serum albumin is composed of more than one moiety. Hewitt (25) was able to isolate two albumins of different chemical composition. Luetscher (24) found, by electrophoretic analysis, that there was comparatively greater reduction of one component of the albumin complex in the serum of the nephrotic patient. It is interesting too, that Bourdillon (26) found that the urine and plasma albumins of nephrotic patients had different molecular weights, an observation confirmed by Longsworth and MacInnes (23). It is possible that this urinary albumin of low molecular weight may correspond to the labile serum albumin fraction (Albumin II), so strikingly reduced in the nephrotic patient.

Fat extraction of nephrotic serum did not produce significant changes in the albumin fractions precipitated with the phosphate precipitant.

#### SUMMARY

I. The serum and plasma protein fractions, separated by sodium sulphate and phosphate precipitants, were studied in premature infants, full-term newborn infants, older infants, and young children. Blood fibrin was also determined.

(a) The blood fibrin levels were found to be constant at all ages, and equal to adult values.

(b) The total serum protein values rise with increasing maturity. Both the albumin and globulin fractions are involved in the increase, but there is a proportionately greater increase in the globulin fraction.

(c) Throughout all of infancy, there is a reduction in certain of the globulin fractions (probably gamma globulin).

II. The serum and plasma protein fractions were determined in children during the different clinical phases of glomerulonephritis.

(a) Plasma fibrin was elevated during the acute stage of the disease and returned to normal

with healing. In the chronic phase of the disease, there is persistent elevation of the fibrin.

(b) In acute glomerulonephritis, there is a slight lowering of serum albumin. In the chronic stage of the disease, both serum albumin and globulin are reduced.

(c) In acute glomerulonephritis, there is an increase in a globulin subfraction (gamma globulin), which returns to its normal level with healing. This globulin subfraction is reduced in value in chronic nephritis.

(d) The relationships between the changes in plasma protein fractions and the alterations in rate of blood sedimentation in glomerulonephritis are discussed.

III. The serum and plasma protein fractions were determined during the different clinical stages of lipoid nephrosis.

(a) Plasma fibrin was elevated during the active phase of the disease.

(b) During the active phase of the disease, the well-known reductions in total protein and serum albumin, and the normal or slightly elevated serum globulin, were encountered.

(c) A reduction in the globulin fraction, presumed to be gamma globulin, was found during the active disease, returning to a normal value with the subsidence of the acute edematous stage. This reduction in the globulin fraction is not of the same nature as that found in young infants. Evidence is presented indicating that the low value for this globulin fraction in the nephrotic, in a large measure results from an altered dispersion of the globulins, caused by the hyperlipemia of nephrosis. Other conditions in which hyperlipemia was present showed similar changes in globulin dispersion.

(d) The reduction of total serum albumin in the nephrotic and in the nephritic patient is largely due to a decrease in a labile subfraction of the albumin.

We wish to express our thanks to Dr. Allan M. Butler for his helpful criticisms and valuable suggestions during the writing of this report.

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# THE PROLONGATION OF THE ACTION OF SUBCUTANEOUSLY INJECTED MEDICINES IN MAN

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The effect of certain metals (Zn, Co, Ni, Cu, Al, Fe, etc.) and tannin, in prolonging the action of various substances injected subcutaneously into animals, has been demonstrated by many workers. Perhaps the first observations in this direction were made by Bertrand and Mâcheboeuf (1) in 1926. They found that the addition of small quantities of Co and Ni salts increased and prolonged the effect of insulin. Extensive work in this field began after 1934. The possibility of prolonging the effect of pituitary gonadotropic hormone (2), insulin (3), the antidiuretic principle of the posterior lobe pituitary extracts (4), histamine (4.a), epinephrine (5), and morphine (6) has been repeatedly shown in various animal experiments.

Notwithstanding the clinical success obtained by prolonging the action of insulin (7) and posterior pituitary extract (8), little work has been done in this important field with regard to other medicines, employed subcutaneously in man. The object of this paper is to show that by the addition of Zn, it is possible to prolong in man the activity of various substances, which greatly differ both in chemical composition and pharmacological action.

## METHODS AND MATERIAL

The drugs employed in the following experiments were: Posterior lobe pituitary extract,<sup>2</sup> epinephrine,<sup>3</sup> and thiamine.<sup>4</sup> Posterior pituitary extract was chosen partly because of the encouraging results attained by its combination with Zn in animal experiments, and partly because we had the opportunity to observe, in a comparatively short period, a number of diabetes insipidus cases. A preliminary report on the therapeutic use of posterior pituitary extract with Zn was published by us recently (8.e). The choice of epinephrine was made because of the contrast between the good results obtained in rabbit experiments (5 and 9) and the negative experi-

ments of Kohn and Bulger (10) in man. Because both posterior pituitary extract and epinephrine are vasoconstrictor agents and hormones, thiamine was chosen to demonstrate that the same principle can be employed on a wholly different substance.

The experiments with posterior pituitary extract were carried out on 3 individuals with normal water metabolism, and 7 patients, of whom 5 were suffering from diabetes insipidus and 2 from polyuria of undisclosed nature. In the present paper, only the observations made on 2 normals and 3 diabetes insipidus patients will be recorded. The investigations with epinephrine were made on 4 hospitalized patients with normal cardiovascular systems (one of them suffering from bronchial asthma), 8 dental patients, and 4 ambulatory patients with bronchial asthma. The subjects in the thiamine experiments were 5 hospitalized patients and 2 physicians, who volunteered for the purpose. Exactly the same amount and concentration of the various substances was injected into the subcutaneous tissue of the gluteal region, on one occasion without Zn and on the other mixed with Zn in a previously determined optimal concentration. This concentration ranged from 0.06 to 0.10 per cent. Zn was added in the form of ZnCl<sub>2</sub>. For reasons to be explained later, the pH of the injections was kept around 5.5. In the case of posterior pituitary extract and epinephrine, the pharmacological action and the presence or absence of unwanted side effects was observed. With thiamine, the rate of excretion in urine was followed.

Throughout this paper in the figures and tables, the following abbreviations will be used: For posterior pituitary extract, PPE; for epinephrine, Adr; and for thiamine, B<sub>1</sub>.

## OBSERVATIONS

### 1. The influence of zinc on the antidiuretic activity of posterior pituitary extract<sup>5</sup>

To study the effect of Zn on the antidiuretic activity of posterior pituitary extract, the rate of urine excretion was observed after the administration of 1000 cc. of tap water, in 2 persons with normal water metabolism and in 3 diabetes insipidus patients.

For persons with normal water metabolism, the arrangement of the experiment was the following:

<sup>5</sup> Dr. I. Strausz (Budapest) cooperated in the posterior pituitary extract experiments.

<sup>1</sup> Resident in Anesthesia, Massachusetts General Hospital, Boston.

<sup>2</sup> Pituisan (Chinoin, Budapest).

<sup>3</sup> Tonogen (Richter, Budapest).

<sup>4</sup> Vitaplex B, (Chinoin, Budapest).

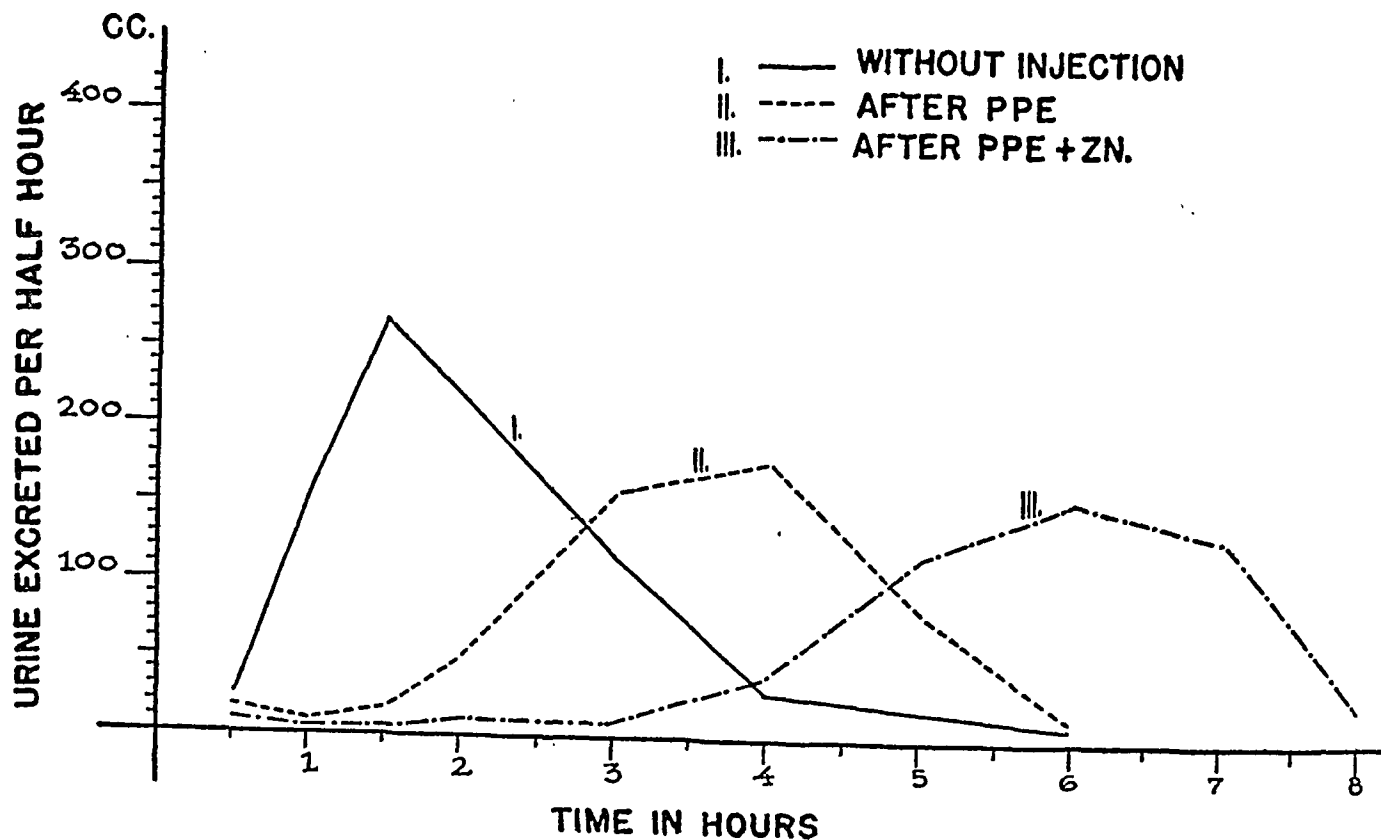


Fig. 1.a

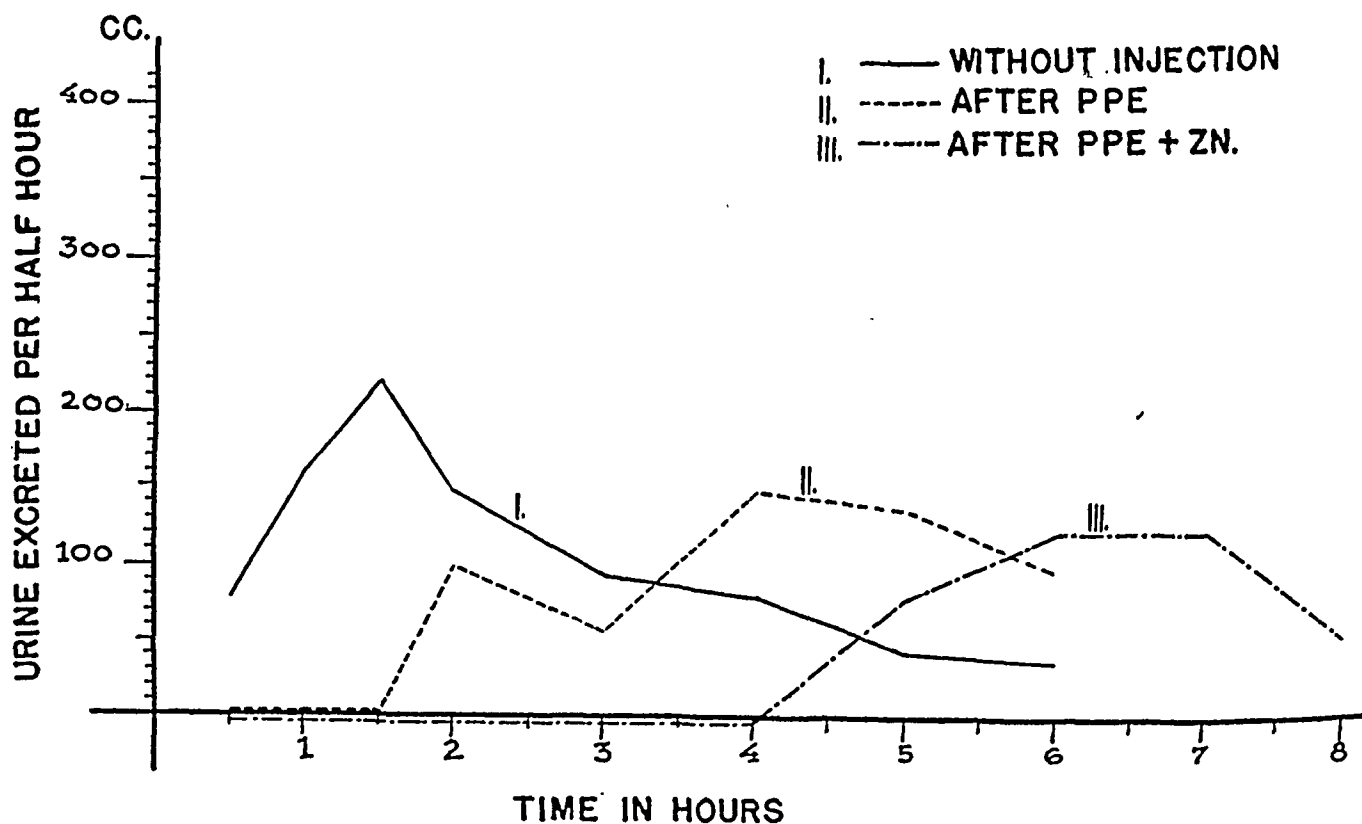


Fig. 1.b

FIGS. 1.a AND 1.b. THE URINE EXCRETION CURVES OF PATIENTS B. S. AND S. N. WITH NORMAL WATER METABOLISM, FOLLOWING THE INTAKE OF 1000 CC. OF TAP WATER

I. Without medication; II. after the subcutaneous injection of 0.4 I.U. of posterior pituitary extract; III. after the subcutaneous injection of 0.4 I.U. of posterior pituitary extract with 0.4 mgm. of zinc.

The individuals were kept in bed 10 to 12 hours before and 5 to 8 hours during the experiments, and no food or fluid was allowed during this period. The bladder was completely emptied before the beginning of the experiment. Immediately afterwards, 1000 cc. of tap water were consumed within 5 to 10 minutes. Urine was then passed  $\frac{1}{2}$ , 1,  $1\frac{1}{2}$ , 2, 3, 4, 5, and 6 hours after the water intake. A few days later the experiment was repeated with the difference that simultaneously with the administration of water, 0.4 I.U. of posterior pituitary extract, diluted to 0.4 cc., was injected in the subcutis of the gluteal region. On yet another occasion, the same amount of posterior pituitary extract was injected with 0.4 mgm. of Zn (in the form of  $ZnCl_2$ ) so that the Zn concentration of the injection was 0.1 per cent. The half-hourly urine excretion produced under different conditions was charted against time (Figures 1.a. and 1.b.).

Figures 1.a and 1.b show that in normal persons, the urine excretion curve reaches its maximum  $1\frac{1}{2}$  hours after water administration. If simultaneously with the water intake, 0.4 I.U. of posterior pituitary extract ( $\frac{1}{25}$  of the therapeutic

dose usually applied) is injected subcutaneously, then the maximum is shifted to the fourth hour, and if the same amount of posterior pituitary extract is injected, well mixed with Zn, the maximum is reached only in the sixth hour. Furthermore, it can also be seen that under physiological conditions, urine excretion starts immediately after the consumption of fluid. While the injection of 0.4 I.U. of posterior pituitary extract hinders any significant urine production for  $1\frac{1}{2}$  hours, the admixture of Zn to the same amount of posterior pituitary extract causes suspension of urine excretion for 3 to 4 hours. This increased activity, incident to the admixture of Zn, can be best

TABLE I

*Total amount of urine collected up to the times indicated*

Time from water intake	B.S.			S.N.		
	Without injection	After PPE	After PPE + Zn	Without injection	After PPE	After PPE + Zn
hours	cc.	cc.	cc.	cc.	cc.	cc.
1	179	28	16	240	0	0
3	901	417	51	802	220	0
6	1018	962	687	1125	1000	410

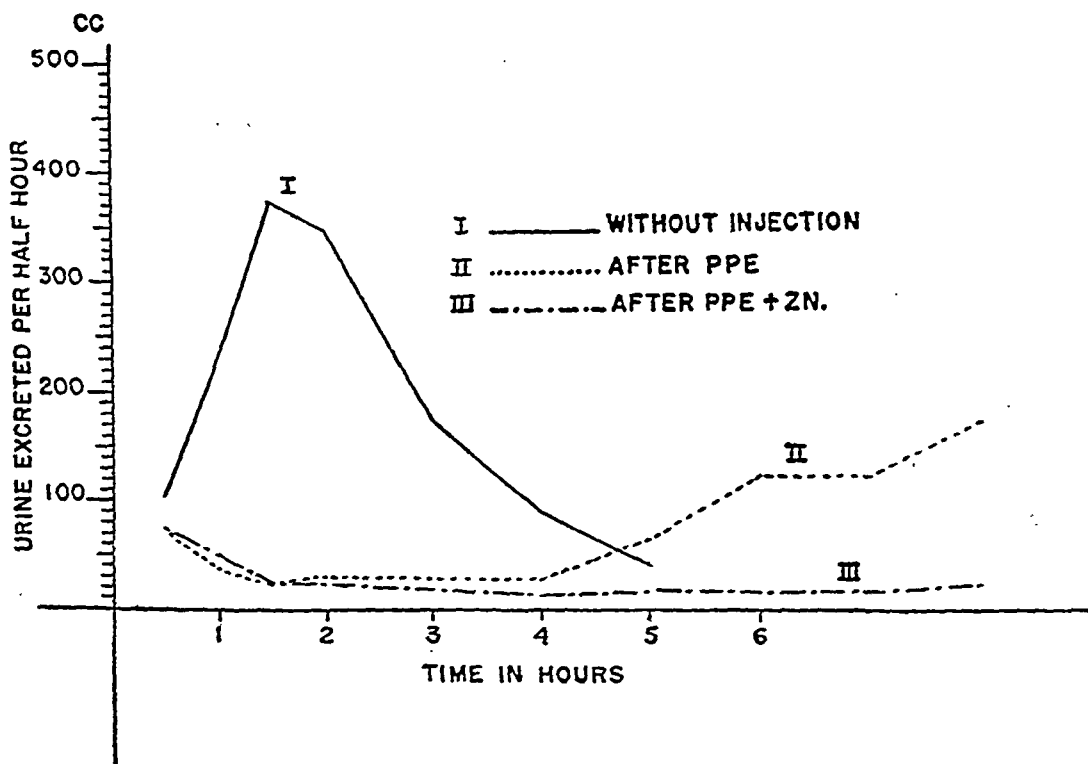


Fig. 2.a



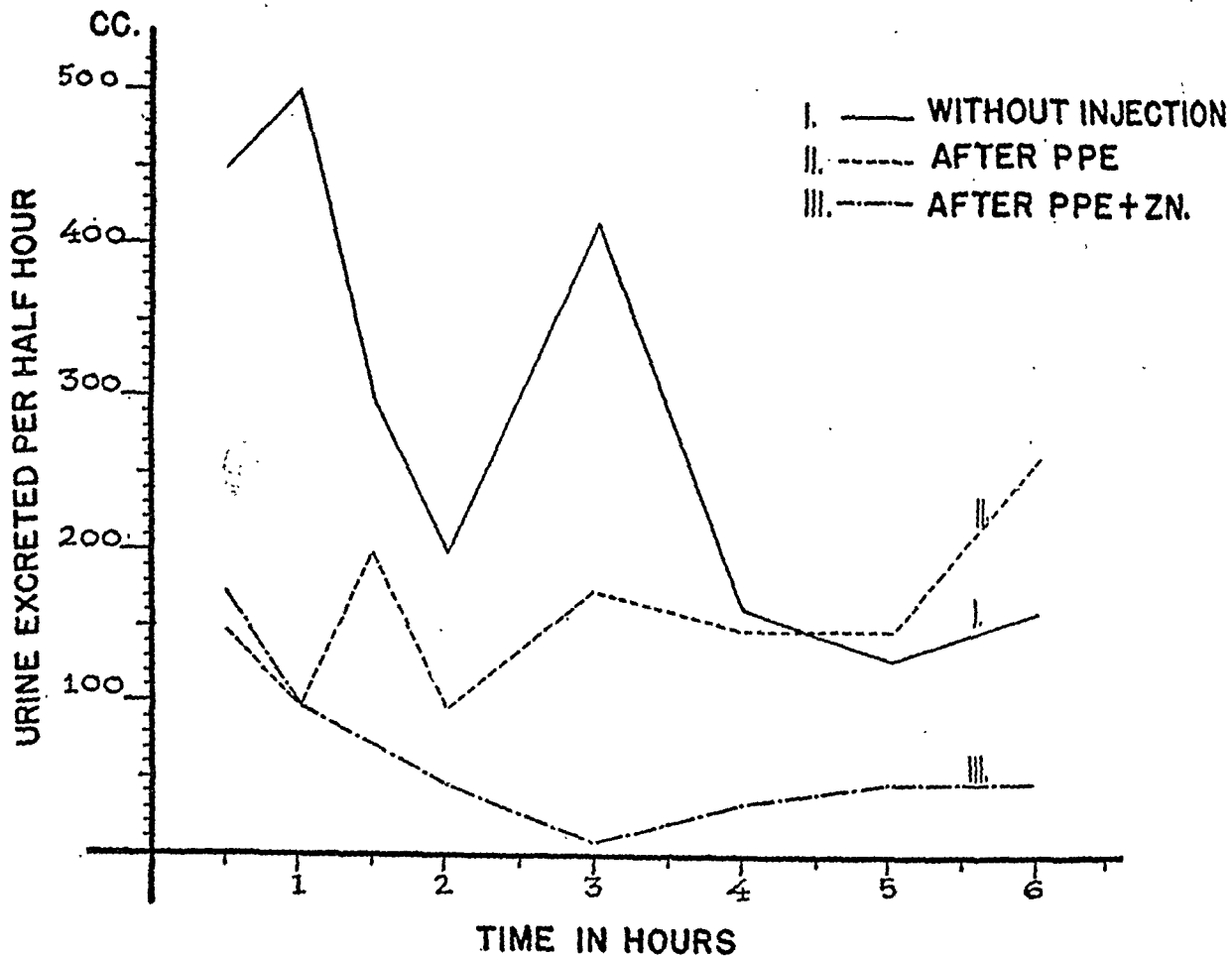


Fig. 2.b

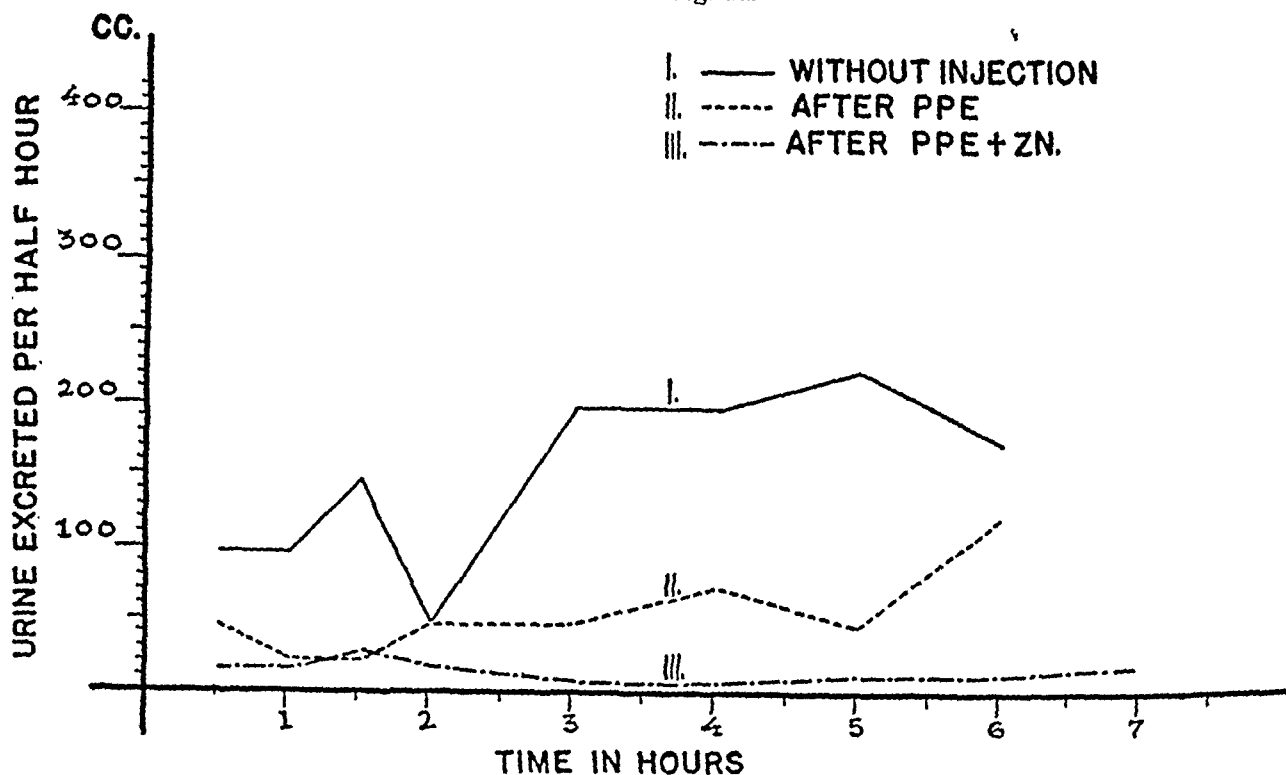


Fig. 2.c

FIGS. 2.a, 2.b, AND 2.c. THE URINE EXCRETION CURVES OF DIABETES INSIPIDUS PATIENTS A. F., M. N., AND H. F., FOLLOWING THE INTAKE OF 1000 CC. OF TAP WATER

I. Without medication; II. after the subcutaneous injection of 10 I.U. of posterior pituitary extract; III. after the subcutaneous injection of 10 I.U. of posterior pituitary extract with 1 mgm. of zinc.

seen if the total urine output for corresponding periods is compared (Table I).

Similar experiments were also carried out on 3 male diabetes insipidus patients whose ages ranged from 19 to 43 years. Their first symptoms appeared 3 to 32 years ago, respectively. No medicines were given for 2 days, food was withheld for 12 hours, and fluid intake for 4 hours preceding the experiment. With the administration of 1000 cc. of water, a dose of 8 I.U. of posterior pituitary extract was injected into the first patient and a dose of 10 I.U. into the second and third patients, in 1 cc. lots, with and without the admixture of 1 mgm. (0.1 per cent) of Zn. The urine output was followed as long as the patients were able to refrain from taking water, but not longer than 8 hours. The results of the experiments are presented in Figures 2.a, 2.b, and 2.c.

Figures 2.a, 2.b, and 2.c show that in every instance there was a great difference in the urine output after drinking 1000 cc. of water, depending on whether it was given without posterior pituitary extract, or with the previous injection of posterior pituitary extract alone or posterior pituitary extract plus Zn. Without posterior pituitary extract, the output of a large amount of urine began immediately after the water intake. The administration of posterior pituitary extract prevented the output of a large quantity of urine for 4 hours in the case of A. F., and for 2 hours in the cases of M. N. and H. F. The pronounced effect of posterior pituitary extract with Zn, however, lasted from 6 to 8 hours. It can also be seen from Figures 2.a, 2.b, and 2.c that the rate of excretion was more uniform with posterior pituitary extract and Zn than with posterior pituitary extract alone.

The effect of the admixture of Zn is still more striking if the total urine output for corresponding

periods is compared (Table II). It can be seen in Table II that while after the administration of posterior pituitary extract the urine output in 6 hours was 30 to 60 per cent of the quantity passed without medication, the amount of urine passed after posterior pituitary extract with Zn was only 10 to 20 per cent.

We had the opportunity to observe the water metabolism of these diabetes insipidus patients for a period of 3 to 8 months (Table III). The

TABLE III  
Comparison of the action of posterior pituitary extract and posterior pituitary extract with zinc

Patient	Period under observation	Average urine excretion in 24 hours			Specific gravity			Duration of pronounced effect	
		Without injection	After PPE	After PPE + Zn	Without injection	After PPE	After PPE + Zn	After PPE	After PPE + Zn
	months	liters						hours	
A. F.	8	9.2	4.8	2.4	1.001	1.003	1.007	3 to 4	8 to 9
M. N.	6	12.5	8.2	4.2	1.001	1.002	1.005	4 to 5	9 to 12
H. F.	3	9.5	9.0	5.0	1.001	1.001	1.004	4 to 5	9 to 15

single daily injection of 10 I.U. of posterior pituitary extract with Zn has proved to be sufficient to reduce the water intake and urine output to from 50 to 30 per cent, and increase the specific gravity of the urine considerably. In the case of A. F., a single injection every second day was sufficient to obtain this result. The single daily injection of posterior pituitary extract without Zn had no significant result, either on the water intake or on the urine output of the patients. An average of 2 to 3 daily injections of posterior pituitary extract was necessary to obtain results similar to those observed after single injections of posterior pituitary extract with Zn. While an antidiuretic effect could be demonstrated for 16 to 36 hours after the injection of posterior pituitary extract with Zn, the effect of the same amount of posterior pituitary extract alone lasted only 4 to 8 hours. After the administration of posterior pituitary extract, unwanted side effects (bowel movement, abdominal cramps, pallor, vertigo, etc.) were quite frequent. No such untoward effects were observed after the injection of posterior pituitary extract with Zn. The only difficulty we encountered in the administration of posterior pituitary extract and Zn was in the case of H. F., in whom some of the injections were

TABLE II  
Total amount of urine collected up to the times indicated

Time from water intake	A. F.			M. N.			H. F.		
	Without injection	After PPE	After PPE + Zn	Without injection	After PPE	After PPE + Zn	Without injection	After PPE	After PPE + Zn
Hours	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
1	400	115	125	950	250	275	200	75	50
3	1400	230	215	2275	900	425	800	250	110
6	1800	675	320	3185	2025	700	2000	750	190

followed by local pain. Since he usually got on quite well by using hypophysis powder intranasally, we decided to reserve the use of the posterior pituitary extract-Zn injections for the occasions when, for some reason (sinusitis, coryza), hypophysis powder could not be administered. By changing regularly the site of the injection, no such difficulty was encountered in the other 2 patients.

Considering the above observations, it might be stated that posterior pituitary extract with Zn, when compared with posterior pituitary extract alone, possesses distinct advantages in the treatment of diabetes insipidus patients. Furthermore, it seems possible that Zn in combination with posterior pituitary extract will prove to be of increasing value in the treatment of certain gynecological, obstetrical, and other conditions where the use of posterior pituitary extract is indicated.

## 2. The influence of zinc on the activity of epinephrine <sup>6</sup>

In preliminary experiments, we found that in man, the optimal Zn concentration of an epinephrine-Zn solution is between 0.06 and 0.08 per cent. A 0.2 per cent epinephrine hydrochloride solution (prepared for us by the pharmaceutical firm G. Richter of Budapest) was used throughout the experiments. The observations were made on 4 hospitalized patients, 2 males and 2 females. The age of the patients ranged between 18 and 40. After 12 hours of bed rest and fasting, the patients received on alternate days 1.6 mgm. of epinephrine, and the same amount of epinephrine with Zn, respectively. The volume of the injection was made up to 1 cc. in all cases and was injected subcutaneously in the gluteal region. Zn was added to the epinephrine solution directly before the administration of the injection. Pulse rate, blood pressure, and blood sugar were determined before, and also 1/2, 1, 2, 3, and 4 hours after the injection. The presence and intensity of subjective symptoms (tremor, palpitation, weakness, etc.) were also recorded. The results are tabulated in Table IV and the combined blood sugar curves are given in Figure 3.

It can be seen from Table IV that the admix-

<sup>6</sup> Dr. L. Kadar (Budapest) cooperated in the experiments with epinephrine.

TABLE IV  
Comparison of the effect of epinephrine and epinephrine with zinc

Patient	Time reckoned from injection	After the injection of Adr.				After the injection of Adr.+Zn			
		Pulse rate	Blood pressure	Blood sugar	Subjective symptoms	Pulse rate	Blood pressure	Blood sugar	Subjective symptoms
	hours			mgm. per cent				mgm. per cent	
A. A.	0	72	100/60	81	—	72	105/70	83	—
	1/2	78	120/60	139	+++	72	110/70	97	—
	1	72	120/60	163	++	72	120/80	123	+
	2	60	100/60	154	+	66	100/80	154	—
	3	66	90/60	95	—	66	100/80	132	—
	4	66	90/60	65	—	66	90/70	90	—
E. E.	0	66	120/60	90	—	66	115/60	83	—
	1/2	78	130/80	168	+++	60	130/70	100	—
	1	78	120/60	175	++	66	140/70	134	+
	2	66	105/60	139	—	72	120/70	161	—
	3	66	105/70	77	—	66	120/70	118	—
	4	66	100/60	70	—	66	110/70	74	—
E. W.	0	72	110/70	83	—	78	100/70	85	—
	1/2	84	130/60	161	+++	84	110/60	123	—
	1	96	126/60	188	+++	84	120/60	166	+
	2	92	115/60	156	++	84	110/70	211	—
	3	80	105/60	85	—	84	110/60	166	—
	4	90	105/60	65	—	84	100/60	95	—
E. P.	0	96	105/80	74	—	90	120/80	72	—
	1/2	114	140/80	129	++	96	115/80	97	—
	1	120	140/70	143	+++	96	125/70	111	—
	2	114	128/70	123	+	96	120/70	150	+
	3	96	110/70	85	+	96	120/70	116	—
	4	90	110/70	65	—	90	110/65	76	—

ture of Zn decreased and postponed the elevation of pulse rate and postponed the blood pressure response caused by the administration of epinephrine. Moreover, there was a significant decrease both in the intensity and duration of the unwanted side effects (tremor, palpitation, etc.).

The behavior of the blood sugar curve (Figure 3) has been particularly interesting. Following the injection of epinephrine plus Zn, the initial rise of the curve was moderate, the maximum was reached later and the elevation lasted longer than after the injection of epinephrine alone. The hyperglycemic effects of epinephrine alone, and of epinephrine with Zn, respectively, can be measured by the surface area bounded by the blood sugar curves and lines parallel with the X axis drawn through the starting point of the curves. The area representing the effect of epinephrine alone was found to be 54.0 units, that of epinephrine with Zn 76.8 units, so that there is a difference of 42 per cent between the two values to the advantage of the latter.

Case A. A. has been a chronic asthmatic patient hospitalized for the frequency of his seizures. He regularly had an attack just after awakening in

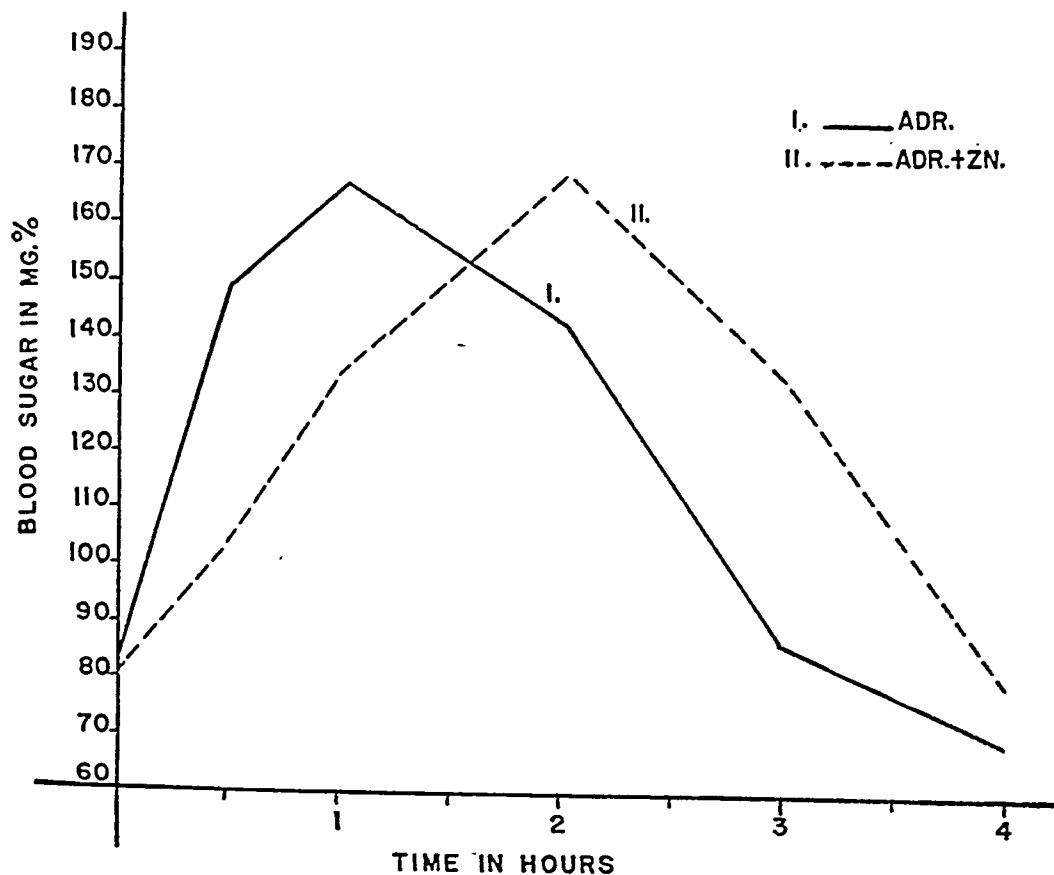


FIG. 3. THE COMBINED BLOOD SUGAR CURVES OF 4 PATIENTS, FOLLOWING THE SUBCUTANEOUS ADMINISTRATION OF 1.6 MGM. OF EPINEPHRINE, AND 1.6 MGM. OF EPINEPHRINE WITH 0.8 MGM. OF ZINC

the morning. The experiments were timed to begin shortly after the attack had developed fully. To our surprise, there was no delay in the onset of the antiasthmatic effect of epinephrine when injected together with Zn. On the other hand, while the effect of epinephrine alone lasted only 3 to 4 hours, epinephrine with Zn was still effective after 6 to 8 hours. On other asthmatic patients, a mixture containing 1.2 mgm. of epinephrine, 5 I.U. of posterior pituitary extract, and 0.08 per cent of Zn proved to have an antiasthmatic effect for 16 to 24 hours.

Another field where epinephrine with Zn has been used effectively is dentistry. The unwanted side effects (pallor, tremor, weakness of the extremities, elevation of blood pressure, etc.) after dental analgesic injections containing epinephrine, are well known. All these symptoms are caused by the general action of epinephrine which is, in these cases, unnecessary, since only the local vaso-

constrictor effect is wanted. By the addition of 0.08 to 0.1 per cent of Zn to the analgesic epinephrine mixture, the unwanted side effects were ruled out completely. A limited number of trials were carried out which not only proved the absence of the unwanted side effects, but also seemed to show a more prolonged local anesthesia.

As already mentioned, there is a distinct discrepancy between the results of Kohn and Bulger (10) and our own experiments. It seems possible that the explanation of this difference might be in the dosage employed. Taking it for granted that the epinephrine preparations used were of equal strength, our dose (1.6 mgm.) was more than three times larger than theirs (0.5 mgm.). A dose of 0.5 mgm. of epinephrine if absorbed rapidly is able to produce a marked hyperglycemia. If, however, its absorption is delayed, or distributed over a longer period of time, the threshold necessary for the production of hyperglycemia will

not be reached, due to the rapid destruction of epinephrine. Since epinephrine is a vasoconstrictor drug which slows up its own resorption to a certain extent, even small quantities of Zn suffice to impede the absorption of a borderline dose of 0.5 mgm., and to prevent a pronounced hyperglycemic effect. On the other hand, if larger doses are applied, the necessary concentration for the production of hyperglycemia will be reached. This coincides with the ultimate objective of the prolongation of the action of medicines, namely: the attainment of a prolonged effect by means of the gradual and evenly distributed resorption of a single large dose.

These preliminary observations on the application of epinephrine with Zn indicate that the admixture of 0.06 to 0.08 per cent of Zn to a subcutaneously injected epinephrine solution diminishes the unwanted side effects and prolongs its period of activity, as measured by the hyperglycemic action.

### 3. The effect of zinc on the urinary excretion of thiamine hydrochloride after subcutaneous injection<sup>7</sup>

It is well known that after the parenteral administration of thiamine, a varying amount of the vitamin injected will be excreted in the urine. The amount excreted depends upon the degree to which the injected person is saturated with thiamine. According to Magyar (11) and Hills (12), less than 18 per cent of the amount injected will be excreted if the patients have latent or manifest thiamine deficiency. It is very interesting that the percentage proportion of the excreted thiamine is fairly independent of the absolute amount injected, and that approximately the same percentage of the quantity injected can be recovered from the urine whether 10 or 50 mgm. were administered parenterally. Magyar (11), Hills (12), and Goth (13) report that the majority of the excreted thiamine can be found in the urine passed within one hour after the administration of the vitamin. We presumed that this rapid excretion was due to the high blood levels which follow parenteral administration, and that by slowing down the rate of absorption by

the admixture of 0.1 per cent of Zn, the amount of thiamine to be excreted might be reduced.

The experiments were carried out on 5 hospitalized patients and 2 healthy persons. The thiamine excretion was tested on 4 of these after the repeated administration of 10 mgm. doses, on 3 after the administration of 50 mgm. doses, and on 1 after the administration of both 10 and 50 mgm. doses. Urine was collected from the experimental persons  $\frac{1}{2}$ , 1, 2, 3, 5, 8, and 24 hours after the injection of thiamine alone or thiamine with Zn. The site of the injection was the gluteal region. The amount of thiamine excreted in the urine was determined by the thiochrome method as modified by Ritser (14).

The results following the administration of the 10 mgm. dose, with and without Zn, are presented in Table V. It can be seen from Table V that

TABLE V  
*Incremental  $B_1$  excretion at the times indicated, following the s.c. injection of 10 mgm.  $B_1$*

Patient	Injection	$\frac{1}{2}$ hour	1 hour	2 hours	3 hours	5 hours	8 hours	24 hours
micrograms								
F. F.	$B_1$	362	744	465	208	178	124	400
	$B_1 + Zn$	38	96	112	651	192	884	224
E. G.	$B_1$	588	1066	777	416	180	86	372
	$B_1 + Zn$	90	450	66	180	260	57	221
M. B.	$B_1$	660	460	256	52	61	44	210
	$B_1 + Zn$	192	205	512	201	88	57	280
N. L.	$B_1$	400	148	180	80	59	61	256
	$B_1 + Zn$	56	60	166	43	80	25	153
T. M.	$B_1$	902	306	480	228	326	140	540
	$B_1 + Zn$	300	280	720	202	36	138	528

after the administration of 10 mgm. of thiamine alone, the intensity of excretion reached its maximum in  $\frac{1}{2}$  hour in 3 cases and in 1 hour in the other 2 cases. If the same amount of thiamine was injected with Zn, the maximal excretion occurred 3 hours after the injection in 1 case, 2 hours after the injection in 3 cases, and 1 hour after the injection in 1 case. The difference between the utilization of thiamine alone and thiamine with Zn can be clearly seen from Table VI in which the total amounts of thiamine excreted during corresponding periods are compared. According to the figures of this table, the quantity of thiamine excreted in a 24-hour period, after

<sup>7</sup> Dr. E. Goth (Budapest) cooperated in the experiments with thiamine.

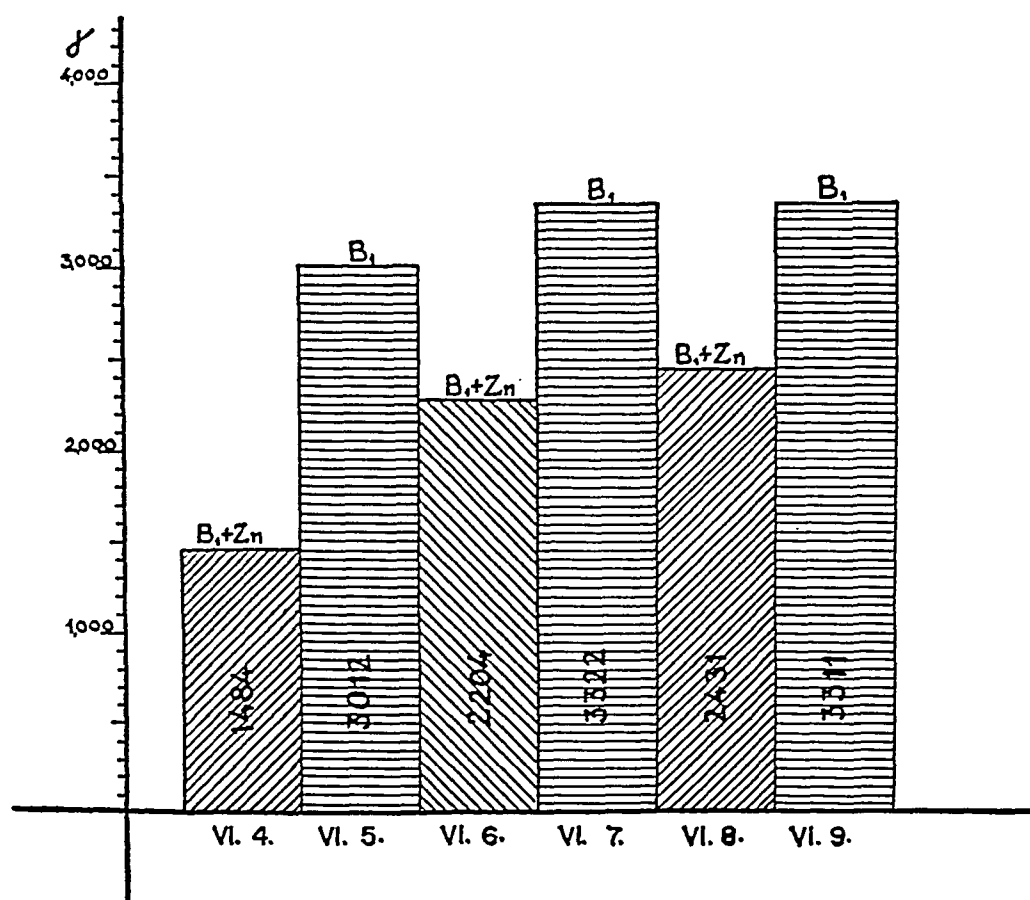


FIG. 4. THE AMOUNT OF THIAMINE EXCRETED IN 24-HOUR PERIODS AFTER THE ALTERNATING DAILY INJECTIONS OF 10 MG.M. OF THIAMINE HYDROCHLORIDE, WITH AND WITHOUT THE ADDITION OF 1 MG.M. OF ZINC

TABLE VI

Total amount of B<sub>1</sub> excreted up to the times indicated, following the s.c. injection of 10 mgm. B<sub>1</sub>

Patient	Injection	1 hour	2 hours	3 hours	24 hours
<i>micrograms</i>					
F. F.	B <sub>1</sub>	1106	1571	1779	2481
	B <sub>1</sub> +Zn	134	246	797	1400
E. G.	B <sub>1</sub>	1654	2431	2847	3485
	B <sub>1</sub> +Zn	540	606	786	1324
M. B.	B <sub>1</sub>	1120	1376	1428	1743
	B <sub>1</sub> +Zn	397	909	1110	1535
N. L.	B <sub>1</sub>	548	728	808	1184
	B <sub>1</sub> +Zn	116	282	325	583
T. M.	B <sub>1</sub>	1298	1778	2006	3012
	B <sub>1</sub> +Zn	580	1300	1500	2200

the injection of 10 mgm. of thiamine with Zn, is considerably (12 to 63 per cent) less than if thiamine alone had been injected. Furthermore, Table VI also shows that a much larger proportion of the total was excreted in the first 3 hours after the injection of thiamine alone, than after the administration of thiamine with Zn. In the case of M. B., we followed up the thiamine excretion after the alternating daily injection of 10 mgm. of thiamine and thiamine with Zn. On every occasion, less thiamine was excreted in the urine after the injection of thiamine and Zn, than after the injection of thiamine alone (Figure 4).

Similar results were obtained if the dose of the administered vitamin was increased to 50 mgm. (Tables VII and VIII).

These results (even if the limitations of the thiochrome method are considered) indicate that the addition of 0.1 per cent of Zn to thiamine in-

TABLE VII

*Incremental  $B_1$  excretion at the times indicated, following the s.c. injection of 50 mgm.  $B_1$*

Patient	Injection	$\frac{1}{2}$ hour	1 hour	2 hours	3 hours	5 hours	8 hours	24 hours
<i>micrograms</i>								
P. P.	$B_1$	10296	3848	1536	4200	648	350	750
	$B_1 + Zn$	1332	3400	4737	1250	960	820	1400
M. T.	$B_1$	4080	1848	1932	620	284	136	450
	$B_1 + Zn$	1420	1668	1340	853	640	1044	800
K. I.	$B_1$	6120	2112	808	317	368	407	1610
	$B_1 + Zn$	2028	3420	7008	1490	270	840	900

TABLE VIII

*Total amount of  $B_1$  excreted up to the times indicated, following the s.c. injection of 50 mgm.  $B_1$*

Patient	Injection	1 hour	2 hours	3 hours	24 hours
<i>micrograms</i>					
P. P.	$B_1$	14144	15680	19880	21928
	$B_1 + Zn$	4732	9469	10719	13899
M. T.	$B_1$	5928	7860	8480	9350
	$B_1 + Zn$	3088	4428	5281	7785
K. I.	$B_1$	8232	9130	9447	11832
	$B_1 + Zn$	6348	14056	15546	16856

jected subcutaneously not only delays its excretion but also diminishes the total amount of thiamine to be excreted in a 24-hour period.

## COMMENT

Since this work has been an attempt to employ Zn for the prolongation of the action of subcutaneously injected medicines, perhaps it is not superfluous to see how this effect of Zn, and other metal salts, is brought about.  $ZnCl_2$  is readily dissolved in an aqueous medium of acid reaction. If however the pH of such a  $ZnCl_2$  solution is shifted towards the alkaline, then above pH 5.5, a fine precipitate forms which consists of  $Zn(OCi)_2$  and  $Zn(OH)_2$ . Furthermore, if an acid solution containing  $ZnCl_2$  is injected into subcutaneous tissue of about 7.4 pH, not only  $Zn(OCi)_2$  and  $Zn(OH)_2$  are formed, but also some of the proteins of the tissue fluids are precipitated by the Zn ions. The precipitate thus formed encloses the

therapeutic agent injected, together with the  $ZnCl_2$  solution. According to the principles of physical chemistry, strong diffusion should start in the direction of the relatively insoluble precipitate. Consequently, the rapid absorption of the injected therapeutic agent will be hindered. Its absorption can only occur gradually, partly by osmosis, and partly by the breaking down of the precipitate. That this is the sequence of events was ingeniously demonstrated by Sahyun (3.d) in experiments made on the ear of the rabbit.

In animal experiments, it was possible to show (9) that the prolonging effect of  $ZnCl_2$  depends not only on the Zn concentration of the injected material, but also on the site of the injection. Provided that the Zn concentration of the injected substance is kept constant, the prolonging effect is the more pronounced, the more dense the subcutaneous tissue where it is injected. The probable explanation of this observation is that in densely woven tissues, the reaction between the injected material and the tissue proteins is more complete and the absorptive surface is also smaller.

The subcutis of man, especially that of the thigh and the gluteal region, is much more suitable for the development of the above reaction than the subcutis of any laboratory animal. Consequently, the amount of Zn necessary to produce maximal prolongation of the effect of posterior pituitary extract and epinephrine is much less (0.1 per cent and 0.06 per cent) in man than in rats (0.5 per cent to 1.0 per cent, Dodds (4.a)) or rabbits (1.2 per cent (Foldes (9))). This is of practical importance because solutions containing less than 0.12 per cent Zn can be injected subcutaneously without any gross local reaction or major discomfort. No general ill effects were observed after the daily administration over a period of more than 6 months. That such small Zn doses should produce any toxic symptoms, even after prolonged administration, seems improbable if we consider that there are 5 to 10 mgm. of Zn present in the average daily diet, and that Zn has been shown to be indispensable in the normal development of certain animals (15).

The fact that on one hand it has been possible to prolong the activity of such widely differing substances as posterior pituitary extract, epinephrine, and thiamine, and on the other hand, that

more than one agent has been found suitable for prolonging the effect of the same substance (*e.g.* tartrate and Zn in the case of posterior pituitary extract) seems to be encouraging as to the future possibilities of this kind of therapy.

# SUMMARY

1. Zinc, in a concentration of 0.06 to 0.1 per cent, has been found to be effective in prolonging the antidiuretic effect of posterior pituitary extract and the hyperglycemic effect of epinephrine.
2. The admixture of zinc also diminished the development of unwanted side effects of both posterior pituitary extract and epinephrine.
3. Posterior pituitary extract with zinc has been successfully employed in the treatment of 3 diabetes insipidus patients.
4. The same zinc concentration delayed and diminished the urinary excretion of thiamine after subcutaneous administration.
5. The prolonging effect of zinc depends not only on its concentration, but also on the site of the injection.
6. The desirability of a systematic study of the prolongation of the effect of other subcutaneously injected medicines is indicated.

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# THE EFFECT OF OPIATES ON THE PAIN THRESHOLD IN POST-ADDICTS

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One of the characteristics of morphine, when used clinically, is the constancy of its action. In the absence of tolerance, a given dose of morphine can be depended on to give a definite result. With one or two exceptions, this is true regardless of the nature or the anatomical location of the pain, unless this is so severe as to be beyond control by the drug. There is some individual variation in the side effects but the main analgesic action is quite constant.

When the pain-threshold-raising action of morphine is studied by the Hardy-Wolff technique (1), taking care to avoid the development of tolerance, a fairly constant response is again observed (2). The effects are not identical in each individual tested, but are of the same order of magnitude.

This report presents results obtained from pain threshold measurements, using morphine and 7 of its derivatives, in a group of men who had been addicted to opiates but who had received no regular doses of these drugs for at least 6 months prior to the study. The post-addiction period ranged from this minimum to a maximum of 8 years.

## METHODS

A Hardy-Wolff apparatus with a 3-second exposure time was used in this study. The lamp was operated from 110 volt A. C. mains through a voltage-regulating transformer, which reduced line fluctuations to an insignificant amount. The brightness of the lamp was controlled by a Variac and the stimulus intensity was measured with a wattmeter connected in the lamp circuit. Without calibration, this does not permit the radiation intensities to be determined in the basic units of calories per cm.<sup>2</sup> per second, but since the results of these measurements are usually expressed as a percentage rise above the pre-injection level, the wattmeter readings give sufficient information for determining time-action curves. Since it has been shown that the wattmeter readings are linear

with radiometer readings, the time-action curves will be identical with those obtained with the radiometer.

All patients used in the test were given preliminary training to familiarize them with the apparatus and the procedure. A few were rejected because of their inability to distinguish the various stimulus intensities. In general, however, no trouble was encountered after the first test. Whenever there was any doubt about the results obtained, the particular test was repeated.

All opiates were given subcutaneously or intravenously and in no case did the patient know the drug or the amount he received. An interval of at least 2 weeks elapsed between successive doses, to avoid complications due to the development of tolerance. At least 5 consistent threshold readings were obtained before administering the drug. After the medication, thresholds were determined at 10 or 15 minute intervals until the values returned to the pre-injection level.

## RESULTS

The pre-injection pain threshold levels were quite comparable with those obtained with non-addicts. Several patients reported that they were "nervous," were unable to stand pain and mental stress, and that these had been contributing factors to their addiction but there was no tendency of the group toward an abnormally low threshold to the type of stimulus used in the present study.

There was, however, an abnormally low response following the administration of morphine and it soon became apparent that to obtain measurable increases in threshold, comparatively large doses would be required. A minimum of 20 mgm. of morphine was chosen, and all results obtained with morphine derivatives are compared to this dosage.

*Morphine.* One of the striking results is the individual variation in the response obtained with a uniform dose (Figure 1-A). In no case was the threshold raising effect of 20 mgm. more than a fraction of that anticipated from the work of Wolff, Hardy, and Goodell (2), and in some cases, a definite lowering of the threshold followed the injection. Seven patients, of a total of

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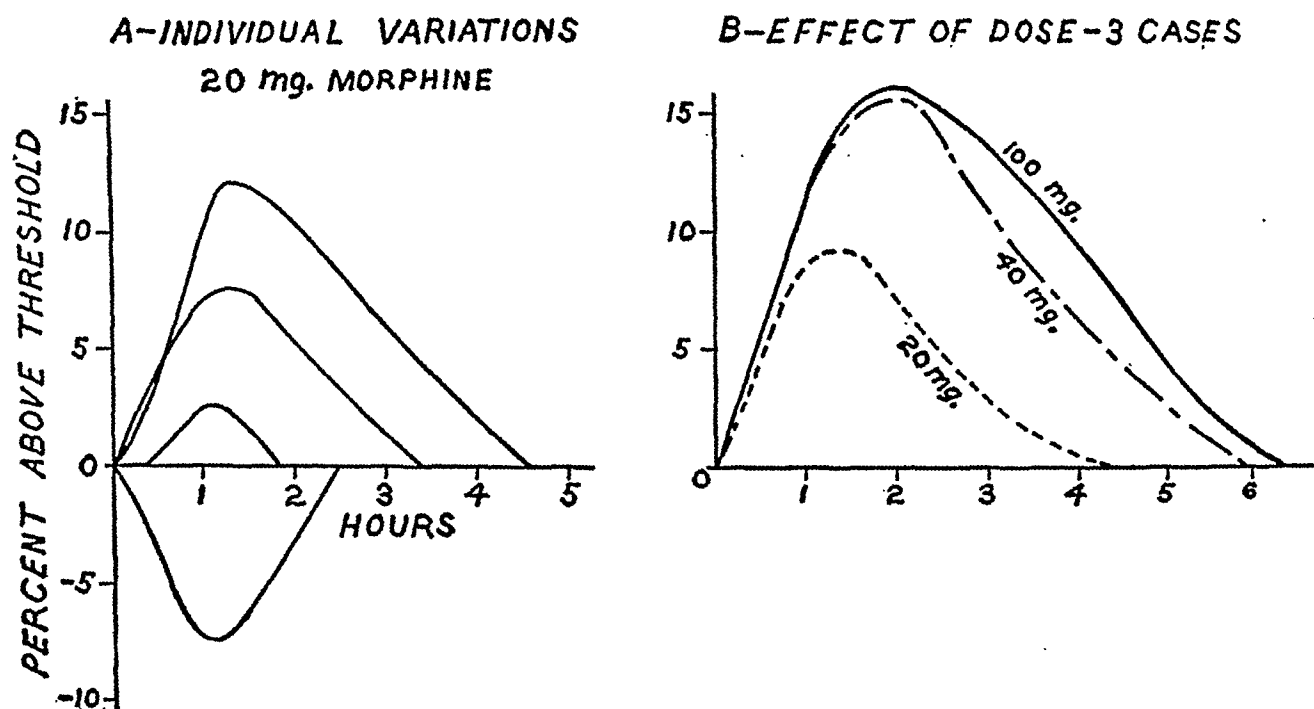


FIG. 1. THE EFFECT OF OPIATES ON THE PAIN THRESHOLD IN POST-ADDICTS

A. Responses from 4 post-addicts showing the large individual variations obtained with this group. On re-test, each case consistently repeated his typical response pattern.

B. Mean curves from a group of 3 cases for 3 dosages. Even with a dose of 100 mgm., the maximum increase in threshold barely exceeded 15 per cent.

57, consistently showed this reversed effect which appears to be genuine, for an essentially similar result was obtained at each re-test. This negative effect is not a true reversal, for in these cases, sufficiently large doses produced a positive threshold-raising action. The response obtained from a single dose of morphine does not appear to be related to the length or intensity of previous addictions, nor to the length of time following the last addiction.

Patients showing this reversed effect were not used in studying the morphine derivatives and because of individual variations, each group receiving a derivative was compared with the same group with morphine.

With larger doses of morphine an increased effect is obtained (Figure 1-B), but a saturation effect is observed (2) and the maximum effect of 100 mgm. is only slightly greater than that of 40 mgm. The total effect in per cent hours, obtained by measuring the area under the curves with a planimeter, increased somewhat, even up to the 100 mgm. dose, because of the prolonged time of action of the larger doses. The total effects in order of ascending doses of 20, 40, and 100 mgm. are 19.3, 49.0, and 58.2 per cent hours respec-

tively. From the curves of Wolff, Hardy, and Goodell (2), the total effect of 20 mgm. would be expected to be about 320 point hours, or 16.6 times that observed in the post-addict. No comparative data are available for the larger doses.

It is of interest to note that the time at which the effect becomes maximal is approximately the same as that found with non-addicts (1.5 hours) and that this time is increased with the larger doses.

The subjective effects of the largest doses were marked, but the smaller doses were tolerated much better than would be the case with non-addicts. Occasional vomiting was encountered with 100 mgm. and 40 mgm. but was absent with 20 mgm. The most consistent finding was myosis. Depression was marked with 100 mgm. but was absent with the smaller doses.

*Codeine.* The work of Himmelsbach (3) indicates that the ability of codeine to satisfy existing physical dependence in man is only 0.19 that of morphine. In cats, Eddy (4) found the analgesic effect to be 0.09 that of morphine. The pain-threshold-raising effect of 100 mgm. of codeine is shown in Figure 2, compared to the effect of 20 mgm. of morphine in the same patients. The

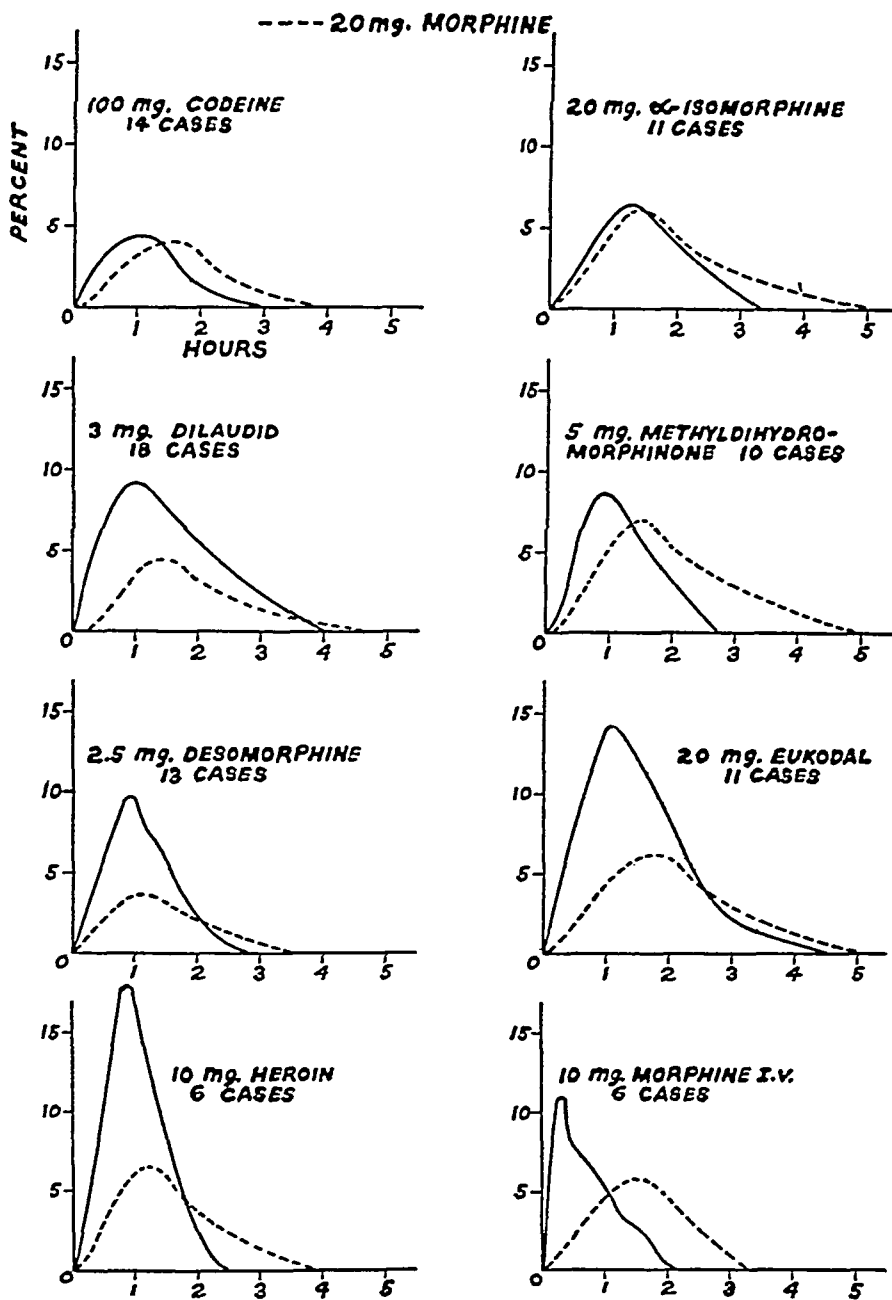


FIG. 2. THE PAIN-THRESHOLD-RAISING EFFECT OF VARIOUS OPIATES IN THE POST-ADDICT

The mean effect of each drug is plotted as per cent of the mean effect of morphine in the same group.

maximum effect of the codeine was quite comparable to that of morphine, but the maximum effect was attained earlier and the duration was shorter. This is surprising since other studies indicate that the duration of codeine action is at

least as long as that of morphine. The total effect of the codeine was 6.7 per cent hours, compared to 7.7 for morphine.

*Alpha-isomorphine.* From substitution studies (3) this drug appears to be 1.4 times as potent as

morphine, while the work on cats (4) shows an analgesic potency ratio of 0.9. The effect of 20 mgm. is compared with morphine in Figure 2. The maximum effects are about equal for the 2 drugs but the alpha isomer has a somewhat shorter time of action. The total effects were 11.2 per cent hours for the alpha isomer and 12.9 for morphine.

*Dihydromorphinone (Dilaudid).* The substitution studies indicate that this drug is 7 times as potent as morphine, while the analgesia studies of Eddy on cats gives a ratio of 4.4. Pain threshold studies in man (2) suggest a ratio of about 10. In Figure 2 is shown a comparison of the effect of 3 mgm. of Dilaudid and 20 mgm. of morphine. With a dosage ratio of approximately 1:7, the total effect of the Dilaudid was 19.0 per cent hours to be compared to only 8.9 per cent hours for morphine. It appears that in the post-addict, Dilaudid is considerably more effective than would be anticipated from other data.

*Methyldihydromorphinone.* Substitution was not entirely satisfactory (5) but indicates that the physical dependence-satisfying action is about 7.2 times that of morphine. The analgesia studies on cats yield a ratio of 7.5 with a duration shorter than morphine. Wolff, Hardy, and Goodell (2) report a ratio of about 4.5 from pain threshold measurements in man, and Lee (6) found a ratio of about 2 from the clinical use of the drug in the relief of pain. Using a dose ratio of 4, it appears that the total effect of methyldihydromorphinone is less than that of morphine, the figures being 12.2 and 15.5, respectively. The maximum effect is somewhat greater than with morphine.

*Dihydrodesoxymorphine-D. (Desomorphine).* Himmelsbach (5) reported an addiction-satisfying ratio of 5 and Eddy (4) an analgesia ratio of 9.4. The clinical studies of Lee (6) indicate a ratio of 11.3. When given in  $\frac{1}{8}$  of the morphine dosage, the total effect was 12.2 per cent hours, to be compared with only 6.8 for morphine. Most of this difference came from the high maximum value which was more than twice that obtained with morphine.

*Dihydrohydroxycodine (Eukodal).* The addiction-satisfying potency is about 0.67 that of morphine (4). Eddy (4) reports an analgesic potency ratio of 0.51. With a dose of 20 mgm., the total effect of 26.0 per cent hours was almost

double the effect of the equal dose of morphine which was 14.0 per cent hours. The maximum effect was more than twice that of morphine and occurred earlier.

*Diacetylmorphine (Heroin).* Eddy (4) reports an analgesic effectiveness in cats of 1.8. When given in a dose  $\frac{1}{2}$  that of morphine, heroin produces a prompt rise in pain threshold. The total effectiveness is 19.7 per cent hours to be compared with 11.6 per cent hours for morphine.

*Intravenous morphine.* There are no comparative data, but an effect having a rapid onset and a short duration would be anticipated. This is actually the case, for a dose of 10 mgm. shows a maximum effect of 11 per cent at 18 minutes, compared to the peak of 5.9 per cent at 1.5 hours, for 20 mgm. given subcutaneously. The total effect was the same from both curves, 10.4 per cent hours.

The results described above are summarized in Table I.

TABLE I

Drug	Effectiveness relative to morphine measured by				Effectiveness relative to morphine on pain threshold in post-addicts		
	Substitution*	Analgesia in cats†	Pain threshold in man‡	Clinical use§	Dose	Maximum effect	Total effect
Morphine	1.0	1.0	1.0	1.0	20	1.0	1.0
Codeine	0.19	0.09	0.10	0.2	100	0.22	0.13
$\alpha$ -isomorphine	1.4	0.9			20	1.0	0.9
Dilaudid	7.0	4.4	10.0		3	13.3	14.2
Methyldihydromorphinone	7.2	10.7	4.5	2.0	5	4.8	3.1
Desomorphine	5.0	9.4		11.3	2.5	20.8	14.3
Eukodal	0.67	0.51			20	2.3	1.9
Heroin		1.8			10	5.6	3.4
Morphine (i.v.)					10	3.8	2.0

\* Himmelsbach (3 to 5).

† Eddy (4).

‡ Wolff and Hardy (2).

§ Lee (6).

The relative effectiveness was calculated from the relative maxima of the time-action curves and also from the total areas under these curves.

## DISCUSSION

The most striking result is the low threshold-raising effect of even a massive dose of morphine. The pre-injection threshold is practically normal but the response following the drug is only a fraction of that obtained in non-addicts. It has been suggested (7) that the first addiction produces certain irreversible changes which might be thought

of as residual tolerance. The present studies confirm this suggestion and indicate that the changes in the pain threshold mechanism are strongly irreversible. Since the effect appears to increase almost linearly with dosage until a saturation level is reached, it appears that there is no barrier to be broken down before the threshold raising action can take place, but rather that the organism has become much less sensitive to this action.

The large individual variations obtained from a standard dose are very interesting and it is unfortunate that nothing has been found in the addiction histories which will correlate with the variations in response. Even those cases who repeatedly show a reduction in pain threshold following morphine have no obviously unusual factors in their make-up which might account for this reversed effect.

In spite of the fact that the post-addict shows a greatly reduced pain threshold response to morphine, the clinical relief of pain is accomplished at this Hospital with no significant increase in morphine dosage over that used with non-addicts. (I am indebted to Passed Assistant Surgeon Leo D. O'Kane for this information.) This fact supports the suggestion (2) that the pain-threshold-raising action is only one part of the process by which relief of pain is achieved, and indeed suggests that this is a relatively unimportant part.

The reduction in pain-threshold-raising effect appears to be somewhat specific, for the response of a group of 12 post-addicts to 10 grains of acetylsalicylic acid, a typical non-opiate, was 76 per cent of that obtained from normals. A further example of this specificity was obtained from a group of post-addicts who showed a reduced response to morphine but an almost normal threshold increase following Demerol. When Demerol was administered regularly, tolerance developed, and the rise in pain threshold was greatly reduced, and has remained so for many months after withdrawal (8). The series of morphine derivatives seemed to provide a suitable tool for studying this specificity, but unfortunately, the results are not entirely satisfactory because of uncertainties in the effects to be expected. A few results, however, appear to be clear-cut. The present results indicate that Dilaudid, Desomorphine, Eukodal, and Heroin are comparatively more effective in the post-addict than other data

would predict. Even with these drugs, the threshold raising effect is only a small fraction of that anticipated from normals.

It should be noted (Figure 2) that all of the more effective drugs are those which are comparatively short acting and which reach a maximum earlier than morphine. If the phenomenon of residual tolerance is a retained ability to rapidly destroy or conjugate opiates and render them innocuous, the more rapidly acting drugs would be expected to appear unduly effective since they would be exposed to this action for a shorter time. This suggestion is supported by the results of intravenous administration, where a doubling of the total effectiveness was observed.

### CONCLUSIONS

When the pain-threshold-raising effect of morphine and its derivatives is tested in post-addicts, a variable but greatly reduced response is found. Results with certain derivatives suggest that this reduced response may be somewhat specific. The abnormally low response could be explained by a retained ability to destroy opiates rapidly. The measurements of pain threshold appear to have little connection with the clinical relief of pain, for this is accomplished in the post-addict with little or no increase in the morphine dosage.

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# SKIN RESISTANCE CHANGES AND MEASUREMENTS OF PAIN THRESHOLD

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When the end-point is reached in the Hardy-Wolff method of measuring pain threshold, there is a sharp change in the subjective experience resulting from the stimulus. When the radiation intensity is below the level of the pain threshold, the sensation is one of heat alone. If the pain threshold is exceeded, there is a sensation of heat with an added factor which has been variously described as "a tweak," "a stab of pain," "a pin prick," or "a threat of tissue damage." The usefulness of this technique lies in the fact that however this end-point is described, it can be duplicated on subsequent trials and after the administration of drugs.

The appreciation and description of this end-point must involve a complex series of neural pathways, including the cerebral cortex. Skin resistance, on the other hand, is primarily under autonomic control, and is only secondarily affected by the cerebrum. Since the stimulus intensity can be adjusted to obtain equal subjective experiences, simultaneous measurements of pain threshold and skin resistance should furnish a means for differentiating between autonomic effects and those involving higher centers. These measurements might be expected to furnish an objective check on the accuracy of the subjective reports, for when the stimulus exceeds the pain threshold, there is a sharp increase in the emotional content of the stimulation and a sudden increase in the magnitude of the skin resistance change would be anticipated. The results obtained from such simultaneous measurements are described in the present report.

## METHODS

Pain threshold measurements were made with apparatus essentially the same as that described by Hardy and Wolff (1). A 3-second stimulus was given at 30-second intervals. The lamp supplying the stimulus was operated from A.C. through a voltage-regulating transformer and controlled by a Variac. Each stimulus in-

tensity was measured by a wattmeter connected in the lamp circuit.

Skin resistance measurements were made from silver chloride electrodes, attached to the palm and dorsum of the hand with collodion. Contact to the skin was made through an isotonic electrode paste. In applying these electrodes, care was taken to avoid injuring the palmar skin, but the dorsum was rubbed vigorously with electrode paste to obtain a low resistance contact. The hand formed one arm of a Wheatstone bridge which was fed from a 1.5 volt cell through a potentiometer. This was adjusted to keep the electrode current below 5 microamperes, thus reducing disturbances due to polarization.

The output of the bridge was fed into a 2-stage, direct coupled amplifier which drove a Westinghouse type PA galvanometer. Photographic recording was on bromide paper. Auxiliary circuits were provided to record the incidence of the stimuli and the subjective reports.

Current was allowed to pass through the electrodes only during the actual measurements, which usually required about 3 or 4 minutes. When the effects of drugs were being studied, a run was made every 15 minutes after drug administration and when the effects lasted more than 2 or 3 hours, new electrodes were applied to eliminate the effects of cumulative polarization.

The pain threshold data were treated in the usual way, the increase over the pre-injection threshold being expressed as a percentage of this threshold and plotted against the time after the injection. The area under each skin resistance change was measured for a 20-second interval with a planimeter. By dividing this area by the base line, the average height of the curve was obtained, and from the bridge calibrations, this was converted to ohms change and to percentage change. Although measurements of maximum change gave results essentially similar to those obtained from the average values, the latter were used in all calculations, since these represent more nearly the total integrated effect of the stimulus.

## RESULTS

The study was carried out on 2 non-addicts and on a group of men who had previously been addicted to opiates but whose addiction had terminated at least 6 months prior to the study. All subjects had had considerable experience with the experimental procedure before being used in the present study. Following each stimulus, a report

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of "warm," "hot," or "pain" (W, H, or P) was given and an appropriate signal was put on the record by the operator.

In general, there is a sharp increase in the magnitude of the skin resistance change when passing

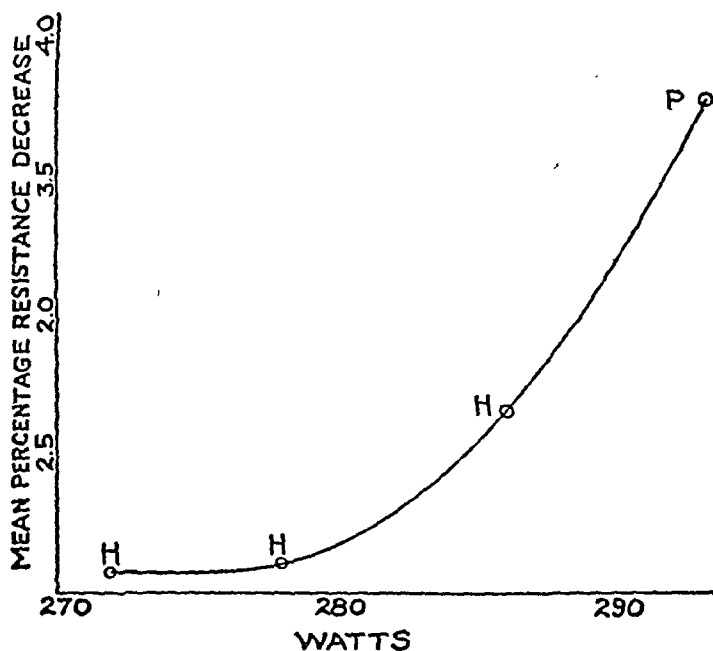


FIG. 1. CHANGE IN SKIN RESISTANCE RESPONSE WITH INTENSITY OF STIMULUS NEAR PAIN THRESHOLD; 124 DETERMINATIONS IN 15 CASES

from an "H" response to a "P." In a few cases, the greatest skin resistance change is observed with the "H" stimulus which immediately precedes that invoking the response. The course of the response from 124 determinations in 15 individuals is shown in Figure 1. This group included 2 men who showed the greatest resistance change to the

"H" stimulus. If these had been omitted, the rise with "P" would have been more pronounced.

When the stimulus intensity was maintained very close to the threshold value so that the subjective response was variable, it was found that a larger resistance response was almost invariably associated with the "P" reports.

Both the magnitude and the duration of the skin resistance change increased when the threshold was exceeded (Figure 2). The time from the incidence of the stimulus to the start of the resistance response was independent of the magnitude of the response. In general, the skin resistance returned to its pre-stimulus value in 20 to 30 seconds, which indicates that the 30-second interval between stimuli cannot be appreciably shortened if independent responses are to be obtained.

When a small dose of morphine (8 mgm.) was given to a non-addict, an increase in the pain threshold was obtained (Figure 3). The change in skin resistance resulting from the "P" stimuli showed a prompt reduction following the injection and this low value was maintained throughout the period of increased threshold. It has been shown (2) that the post-addict shows a greatly reduced response to the pain-threshold-raising effect of morphine. An 8 mgm. dose of morphine produces no significant increase in pain threshold, but is followed by a prompt reduction in the magnitude of the skin resistance response which is quite comparable to that obtained with non-addicts. In 2 experiments, this reduction persisted for 13 and 14 hours, respectively, at which time

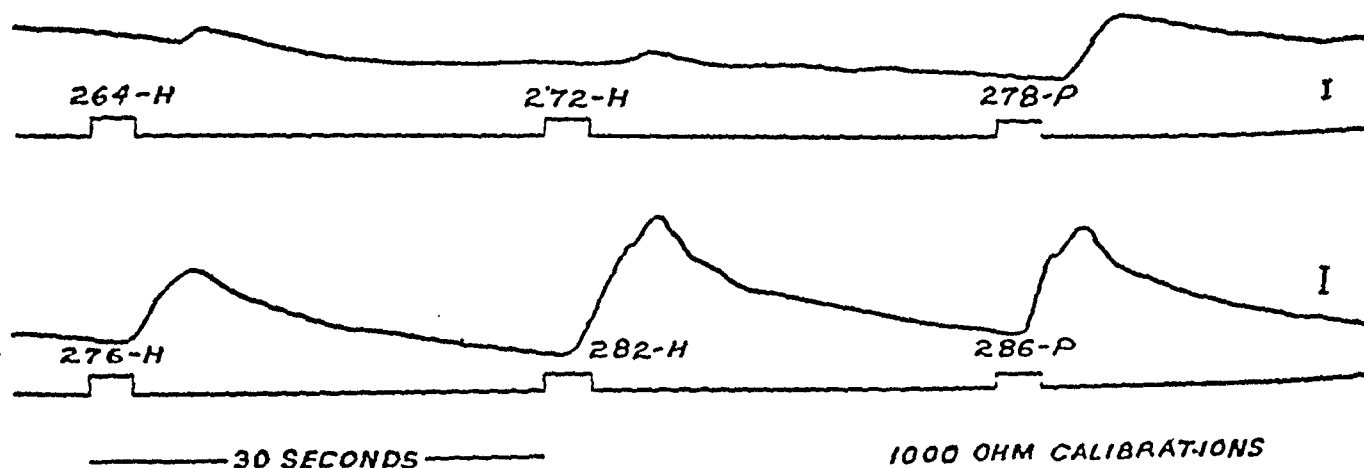


FIG. 2. CHANGES IN SKIN RESISTANCE AT THRESHOLD

al skin resistance responses. The stimulus intensity and the subjective response are recorded at each elevation of the signal line which marks the incidence of the stimulus.

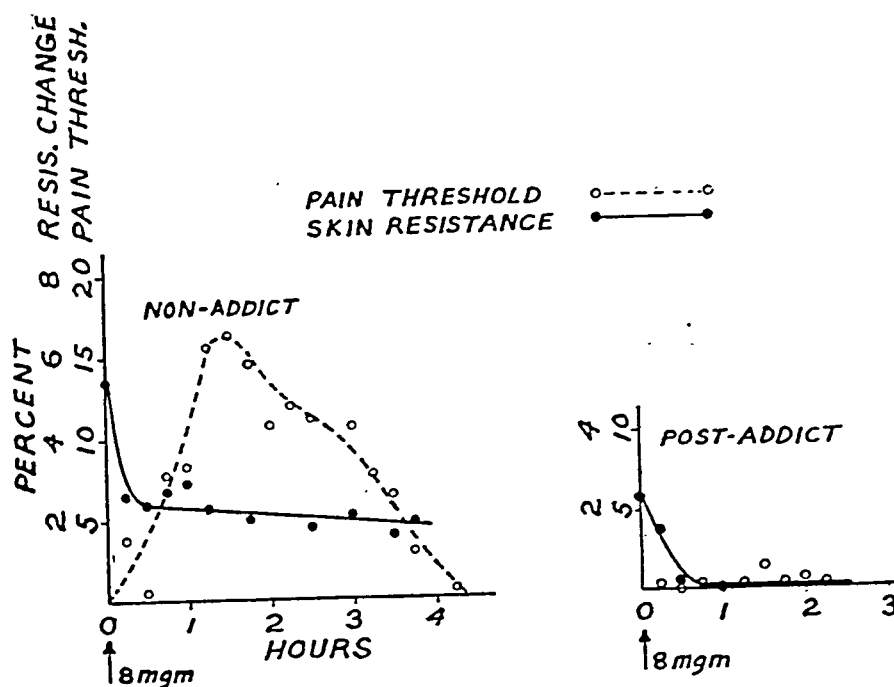


FIG. 3. EFFECT OF MORPHINE ON PAIN THRESHOLD AND SKIN RESISTANCE

Typical changes in the magnitude of the skin resistance response for "P" stimuli following morphine. In both cases, there is a decrease in the skin resistance response but in the post-addict, this is not accompanied by a change in the pain threshold.

the effect started to increase toward the original values. When larger doses (20 to 100 mgm.) were given to the post-addict, an increase in pain threshold was obtained and the reduction in the skin resistance change was so marked that no response could be obtained with the recording system at maximum sensitivity.

When an increase in pain threshold was obtained with acetylsalicylic acid instead of with morphine, there was no reduction in the magnitude of the skin resistance response, even though the maximum increases in pain threshold were comparable. Post-addicts showed some reduction in skin response following 100 mgm. of codeine, but this reduction was less than that obtained with 20 mgm. of morphine, which produces a comparable rise in pain threshold. In the post-addict, the reduction in skin response following 100 mgm. of Demerol was at least as great as that produced with 20 mgm. of morphine, but the pain-threshold-raising effect of the latter was somewhat greater.

#### DISCUSSION

It appears that the skin resistance response cannot be used as an objective measure of the end-

point in the determination of pain thresholds, for the response following a "P" report is not invariably greater than with weaker stimuli. In a group, there is a strong tendency for a greater response with a "P" report, which bears out the subjective reports that above threshold, the stimulus has an emotional content considerably greater than stimuli only slightly below threshold. It seems probable that the maximal responses occasionally obtained with the strongest sub-threshold stimuli are due to subjective uncertainty and confusion, producing an autonomic disturbance which is reflected in the increased skin resistance change. When the strength of the stimulus is increased, the uncertainty disappears and the skin response becomes smaller.

Simultaneous measurements of skin resistance and pain threshold are of value, however, in differentiating various aspects of drug action. The action of morphine on that portion of the autonomic system involved in controlling the skin resistance appears to be comparable in post-addicts and normal subjects. However, when the cerebrum becomes involved in the recognition and in-

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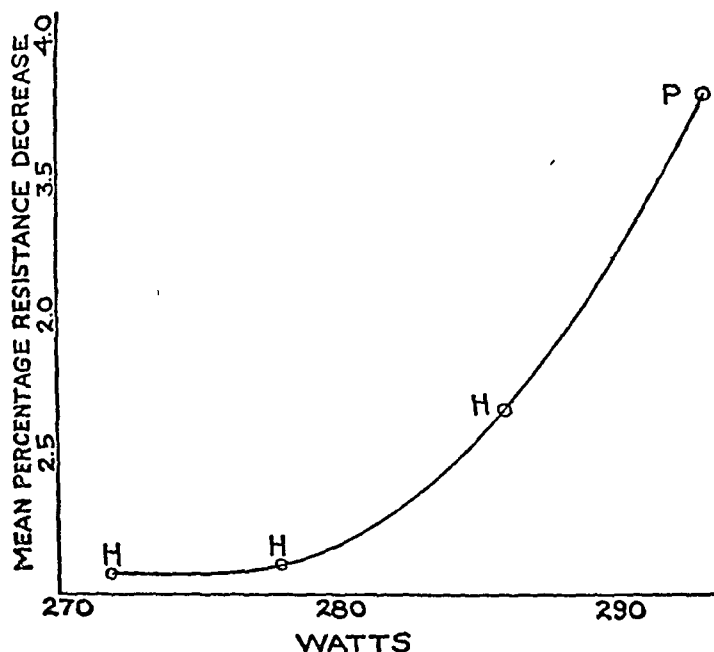


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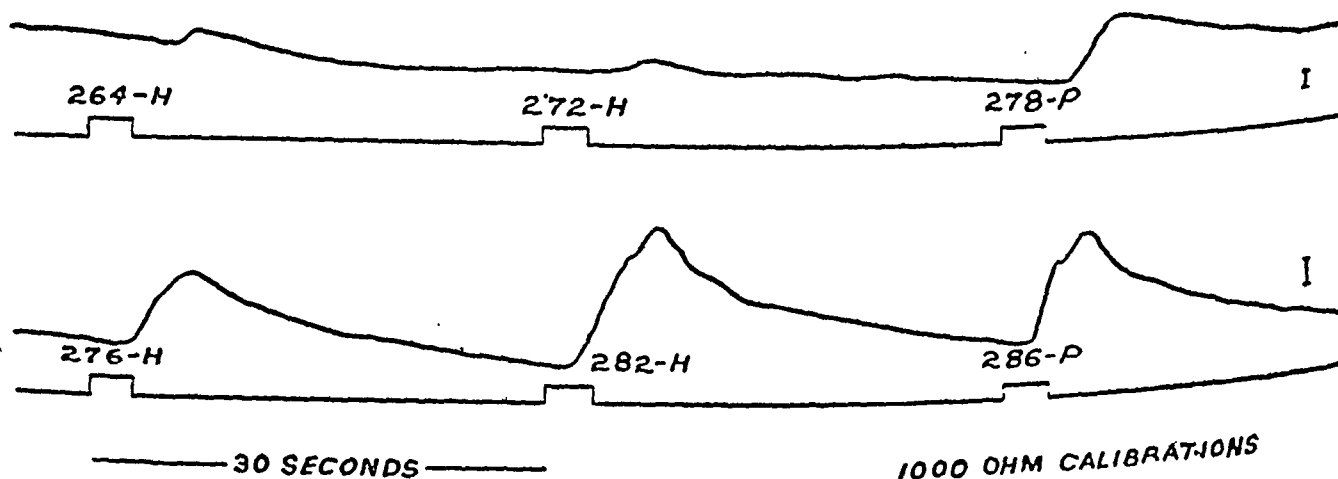


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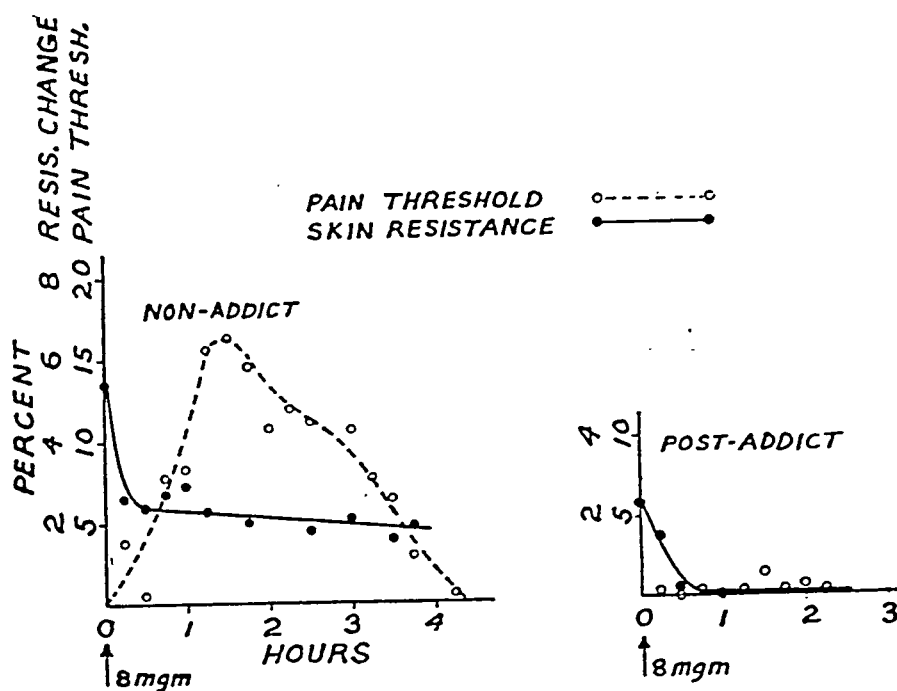


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Simultaneous measurements of skin resistance and pain threshold are of value, however, in samples and the antedifferentiating various aspects of drug action on that portion of the autonomic system involved in controlling the capacity of the skin resistance appears to be comparable to the best of three samples were made by and normal subjects. However, 1.6 per cent potassium permanganate becomes involved in the

terpretation of the pain threshold, there is a distinct difference between the 2 groups.

The fact that morphine produces a reduction in skin response in the post-addict, comparable to that obtained in non-addicts, is of some importance in explaining the clinical relief of pain. It has been shown (2) that the pain-threshold-raising-effect of morphine is greatly reduced in the post-addict, yet clinical relief of pain is obtained with no increase in dosage. The results of the present study indicate that in the post-addict, the mechanisms involved in the skin resistance response are intact, while there is a permanent change in those involved in the recognition of pain threshold. It is interesting that the duration of the effect on skin resistance is about 14 hours, in good agreement with the value given by Himmelsbach (3) for the duration of addiction-satisfying action.

Wolff, Hardy, and Goodell (4) have suggested that the relief of pain by opiates involves at least 2 mechanisms: An increase in pain threshold, and a reduction in the reaction to a recognized pain. They have further shown (5) that the pain threshold reaction is quite independent of this "alarm reaction."

The present studies corroborate these views and suggest that with morphine, the reduction in the alarm reaction is the more important factor. Acetylsalicylic acid appears to relieve pain almost entirely through its pain-threshold-raising action. Codeine exhibits both actions, but the effect on skin resistance is considerably smaller than with morphine. This is in accord with its recognized ability to relieve pain without producing the euphoria and sense of well-being commonly encountered with morphine. The marked effect of Demerol on the skin resistance changes correlates with subjective reports, for this drug produces changes in reaction patterns which are comparable to those of morphine.

## CONCLUSIONS

In general, the skin resistance change produced by the radiant stimulus used in the Hardy-Wolff technique is increased with a subjective report of pain. There are some variations, so this cannot be used as an objective measure of the end-point.

In non-addicts and in post-addicts, there is a reduction in the skin resistance response following a dose of morphine.

There is some reduction following codeine but this is not as pronounced as with morphine.

The reduction following Demerol is at least as great as that observed with morphine.

Following morphine, the reduction in skin response is maintained considerably beyond the pain threshold effect.

The reduced skin resistance response is probably associated with a reduced pain appreciation, which offers an explanation of the clinical relief of pain in the post-addict.

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# A STUDY OF THE VOLUME OF THE BLOOD IN CONGESTIVE HEART FAILURE. RELATION TO OTHER MEASUREMENTS IN FIFTEEN PATIENTS

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While the volume of the blood in health is quite constant and closely related to the size, age, and sex of the individual, it has long been recognized that in congestive heart failure, this relationship is disturbed. Therefore, investigations of the volume of the blood in this clinical condition should throw light on the mechanism of normal control of the volume of the blood as well as on the nature of the physiological disturbances involved in heart failure itself. Studies of this nature, summarized by Gibson and Evans (1), indicate that there is an increase in the volume of the blood in this condition. While this generality is well supported, there is a striking variability in individual cases. It appeared likely that this variability might be accounted for if quantitative measurement of factors which might influence blood volume were made at the time of the determination of the blood volume. Since the association between anoxemia and compensatory polycythemia is well established, measurements of arterial and venous oxygen were made and it proved possible to show a rough quantitative relationship between anoxemia and cellular increase. On the other hand, while the statement has been made (1, 2) that there is a positive relationship between the venous pressure and the change in blood volume in congestive heart failure, it was not possible to find such a relationship *within* this group of severely congested cardiac patients.

Although a number of the lines of investigation pursued did not lead to the establishment of any simple correlation with plasma or cell volume changes, these data presented are of interest, not only in themselves, but also because certain interesting relations obtain between them. The results of these studies conducted on cardiac patients are therefore presented in full.

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## MATERIAL

Fifteen patients were selected who had clinical evidence of severe congestive heart failure (class IV). All were dyspneic or orthopneic, all but one had pitting edema, all but one had hepatomegaly, most had chronic passive congestion of the lungs, a few had hydrothorax, several had ascites. The clinical findings in these patients are summarized in Table I. Every effort was made to select uncomplicated cases, who were untreated, or who were in a steady state despite digitalis. Etiology of the heart disease in these cases included rheumatic heart disease, arteriosclerosis, hypertension, thyrotoxicosis, syphilis, and heart disease secondary to pulmonary disease. The duration of failure ranged from a few weeks to several years. Patient number seven had evidence of a moderate degree of renal insufficiency and he also had hemoptysis. None of the others had evidence of chronic blood loss. Five patients were female and ten were male. The average age was 54.2 years, with one subject in the third decade, five in the fourth decade, four in the fifth, and five in the sixth decades.

## METHODS

The patient was brought to the laboratory in bed in the post-absorptive state. The bed was adjusted to as flat a position as the subject could tolerate, and he was allowed to rest in this position for more than thirty minutes. Then the blood volume was determined by the method of Gibson and Evans (3). Predictions of blood, plasma, and cell volumes, normal for these patients were based on Gibson and Evans data (4), using the height of the subject, because edema rendered the weight an unsatisfactory reference value. Predicted values were subtracted from observed values, and the difference expressed as a percentage of the normal.

The same venipuncture made for the injection of the dye was used to determine the arm to tongue circulation time (5) and the venous pressure (6). The venous pressures were referred to a point six centimeters below the Angle of Louis. A period of rest followed, then samples of blood were taken from the radial artery and the antecubital vein simultaneously in oiled syringes and stored over mercury with refrigeration. Dry potassium oxalate was used as the anti-coagulant. The vital capacity was determined with a spirometer, recording the best of three trials. Several hematocrit determinations were made by the wet technique of Gibson (3), using 1.6 per cent potas-

TABLE I  
Observations on 15 patients with chronic congestive heart failure (Class IV)

Case number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Initials	C. C.	S. W.	E. D.	F. S.	H. G.	C. B.	L. M.	J. H.	H. M.	G. C.	A. M.	W. D.	V. G.	A. P.	W. D.
Diagnosis	Hypertensive and arteriosclerotic heart disease, coronary artery fibrillation	Heart disease secondary to pulmonary fibrosis and emphysema, 1	Hypertrophic and arteriosclerotic heart disease	Pulmonary heart disease, fibrosis	Systolic heart disease, aortic insufficiency	Rheumatic and hypertensive heart disease, mitral stenosis and aortic regurgitation, associated renal insufficiency	Rheumatic and hypertensive heart disease, mitral stenosis and aortic regurgitation, associated renal insufficiency	Hypertensive and arteriosclerotic heart disease, coronary artery fibrillation	Rheumatic heart disease, mitral and aortic stenosis and insufficiency, aortic fibrillation	Arteriosclerotic heart disease, coronary artery fibrillation	Arteriosclerotic heart disease, coronary artery fibrillation	Hypertrophic heart disease, aortic fibrillation	Arteriosclerotic heart disease, coronary artery fibrillation	Rheumatic heart disease, mitral stenosis and aortic regurgitation	Arteriosclerotic and hypertensive heart disease, coronary artery fibrillation
Physical data															
Age years	58	65	58	50	61	41	47	52	46	66	59	50	65	35	61
Sex	M	M	F	M	M	M	F	F	F	M	M	F	M	M	M
Height cm.	172	158	163	163	169	176.5	157	148	152	165.5	164	165	167	172	171
Weight kg.	91.2	73.8	62.6	61.0	85.0	75.6	65.8	66.6	83.4	67.2	64.8	62.0	63.6	62.0	91.0
Surface area, sq. M.	2.07	1.74	1.69	1.64	1.94	1.90	1.65	1.59	1.78	1.73	1.69	1.66	1.66	1.81	2.01
Clinical manifestations															
Duration of failure	3 to 6 months	weeks	9 months	5 weeks	2 months	10 months	repeatedly 14 years	1 to 2 months	6 years	months	2 to 3 months	2 months	2 months	years	3 weeks
Edema	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Ascites	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hepatomegaly	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Hydrothorax	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Dyspnea	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Orthopnea	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Pulmonary congestion	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Urine: albumen casts RBC/HPF															
	++	+	++	++	+	++	++	++	++	+	++	+	++	++	+
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	2 to 3	2 to 3	0	0	0	0	0	0	0	0	0	0	0	0
Digitalized															
	0	0	amount?	0	partially	fully	fully	0	?	0	0	0	0	none for 1 week	0
Other therapy															
	0	0	0	0	0	NH <sub>4</sub> Cl	salycan	0	?	0	0	0	0	0	0
Evidence of blood loss															
	0	0	0	0	0	hemop.	0	0	0	0	0	0	0	0	0
Physiological measurements															
Blood volume, per cent change	+37.	+110.	+28.	+19.	+47.	+45.	+42.	+69.	+98.	+45.	+38.	+34.	+41.	+25.	+14.
Plasma volume, per cent change	+40.	+76.	+47.	+13.	+38.	+81.	+3.	+67.	+91.	+61.	+15.	+44.	+45.	+31.	+14.
Cell volume, per cent change	+34.3	+151.	0	+27.4	+55.3	0	+100.	+71.	+106.	+25.6	+65.3	+19.4	+36.	+19.	+14.6
Arterial systolic pressure	198	130	165	100	154	198	225	154	140	130	145	124	180	122	180
Arterial diastolic pressure	134	70	70	80	80	145	140	125	85	151	95	58	110	82	140
Venous pressure, mm. H <sub>2</sub> O	160	154	267	215	254	197	237	175	192	151	178	144	130	270	244
Arm-longue time seconds	27.5	20.0	13.5	53.2	29.9	34.3	32.0	57.0	23.5	151	178	13.0	110	35.0	36.8
Vital capacity liters	1.40	0.7	1.15	1.20	1.75	2.05	1.00	1.00	1.67	1.60	1.78	2.00	1.10	2.50	1.35
X-ray T.D./L.D.	0.655	0.604	0.564	1.20	1.75	0.696	0.750	0.744	1.67	1.60	1.78	0.572	1.10	2.50	0.692
Arterial blood															
CO <sub>2</sub> content	44.15	70.6	48.28	39.63	37.97	30.19	47.83	40.90	45.01	52.7	62.82	47.15	48.79	33.62	44.35
O <sub>2</sub> content	16.43	10.44	11.72	17.64	18.15	12.18	21.20	14.75	15.51	13.44	8.96	14.21	12.63	14.68	15.97
O <sub>2</sub> saturation	78.8	46.9	88.7	85.5	93.3	88.9	90.6	82.9	80.2	83.2	41.8	92.5	68.7	94.0	85.9
Venous blood															
CO <sub>2</sub> content	47.21	71.6	48.47	42.60	49.89	34.09	57.04	45.79	46.11	57.01	63.38	48.78	49.53	42.53	47.47
O <sub>2</sub> content	10.50	8.55	10.76	11.09	9.23	8.55	5.69	9.57	14.08	6.62	7.73	11.65	10.02	2.57	11.99
O <sub>2</sub> saturation	51.8	39.5	73.2	56.3	47.8	63.4	24.4	56.9	70.0	40.1	35.4	75.0	53.6	16.2	65.1
Miscellaneous															
Capillary unsaturation	7.09	12.5	2.7	5.8	6.4	3.2	9.9	5.2	4.4	6.3	14.2	2.5	7.2	7.1	4.5
Hemoglobin	15.1	16.16	9.9	15.4	15.16	10.23	17.44	13.2	14.99	12.3	16.3	12.3	13.7	12.1	13.08
Volume packed RBC	43.4	53.3	31.2	47.2	47.5	33	55.9	40.5	41.7	38.1	53.5	34.9	42.8	42.3	49.5
Plasma CO <sub>2</sub> C.P.	60	79	55	51	51	33	55.9	40.5	55	38.1	74	51	55	49	57
Plasma protein T.P.	5.5	5.7	5.7	6.0	6.25	5.8	7.1	5.5	6.1	5.5	5.3	5.7	6.1	5.9	6.0

VOLUME OF THE BLOOD IN CONGESTION

TABLE II  
Determinations repeated after clinical change

Patient number	12		13		14		15	
	F		M		M		M	
	Improved		Improved		Worse		Improved	
	18 days		13 days		6 days		4 days	
Sex								
Clinical change								
Interval between determinations								
Venous pressure, mm. of H <sub>2</sub> O	144	107		64	109	270	244	114
Arm to tongue time, seconds	13	13			26	35	37	33
Vital capacity, liters	2.0	2.6	68.7	90.3	2.9	2.5	1.4	1.7
Arterial O <sub>2</sub> saturation, per cent	92.5	92.5	53.6	60.6	97.1	94.0	85.9	91.4
Venous O <sub>2</sub> saturation, per cent	75.0	60.0	7.2	5.2	62.3	16.2	65.1	70.7
Capillary unsaturation, volumes per cent	2.5	3.9	13.7	16.3	3.6	7.1	4.5	4.2
Hemoglobin, grams	12.3	12.5	43.2	47.0	13.8	12.1	13.7	17.4
Volume packed red cells, per cent	35.4	38.9	6.1	6.8	46.5	42.2	44.8	51.8
Plasma proteins, grams per cent	5.7	6.2			6.8	5.9	6.0	4.7
Blood volume, per cent change	+34	+21	+41	+21	+8	+25	+14	-8
Cell volume, per cent change	+19	+18	+36	+27	+13	+19	+15	+7
Plasma volume, per cent change	+44	+23	+45	+16	+4	+31	+14	-20
Weight, kgm.	62	57			56	62	91	79

sium oxalate. The arterial blood pressure and the height and weight were determined.

Analyses of the blood samples for oxygen and carbon dioxide content and oxygen combining power were made (7) and the hemoglobin content calculated. Capillary unsaturation was calculated from Lundsgaard's (8) formula

$$\frac{A + V}{2}$$

where  $A$  and  $V$  are the arterial and venous unsaturation respectively. The carbon dioxide combining power (9) and the plasma total protein (micro-Kjeldahl) were determined. In nine patients, satisfactory teleroentgenograms of the heart were taken. These were measured to obtain the internal diameter of the chest at the level of the fourth costochondral junction and for transverse diameter of the heart, taking the horizontal distance from the outermost right to the outermost left border. The ratio of the transverse diameter of the heart to the internal diameter of the chest was calculated.

The data obtained are presented in Table I. In several of the patients, the whole procedure was repeated after a clinical change in the patient, and the relevant data showing these changes are presented in Table II.

### RESULTS

The blood volume was increased in all cases and in the great majority, the increase was substantial. The mean increase over predicted was  $46 \pm 27^2$  per cent. The plasma volume was increased with a mean of  $44 \pm 27^2$  per cent for the group as was the cell volume with a mean of  $\pm 43^2$  per cent. While the mean increase in the cell volume is greater than the increase in the plasma volume, in the light of the variability

<sup>2</sup> One standard deviation not corrected for small size of sample.

within the group, there is no statistical significance in the difference between these means. Even when the more sensitive comparison in each individual case is made, the mean difference between the cell volume and the plasma volume is only plus 5.5 per cent. This mean is not significantly different from zero by Fisher's method of  $t$  (11).

The mean of the venous pressures of the group is  $203 \pm 45^2$  mm. of water in the fourteen cases where measurements were made. In nearly all the cases, right upper quadrant pressure for about ten seconds produced a rise in venous pressure. This test has often been discussed in connection with congestive heart failure, but in this study, it was performed primarily for the purpose of assuring a free connection between the manometer and the venous circuit.

The venous pressure was compared with the blood volume, the plasma volume, and the cell volume, by graphic and statistical methods. (Figures 1 and 2 and Table III.) There is no significant correlation between the blood volume or the plasma volume and the venous pressure. The interpretation of this statement must be strict if it is to have meaning. These patients all suffer from severe congestive heart failure. If a group of cases ranging from normal through mild insufficiency to severe heart failure were studied, there would in all probability be a high correlation between venous pressure and the blood volume or plasma volume. The finding means simply that



within a group of patients with severe failure there is no correlation between venous pressure and blood volume or plasma volume. There is a negative correlation of moderate significance between the cell volume and the venous pressure.

The circulation time for the group is increased to a mean of  $31 \pm 18^2$  seconds. Two patients (case number 3 and case number 12) had short circulation time from arm to tongue. Both of these had hyperthyroid heart disease. The cir-

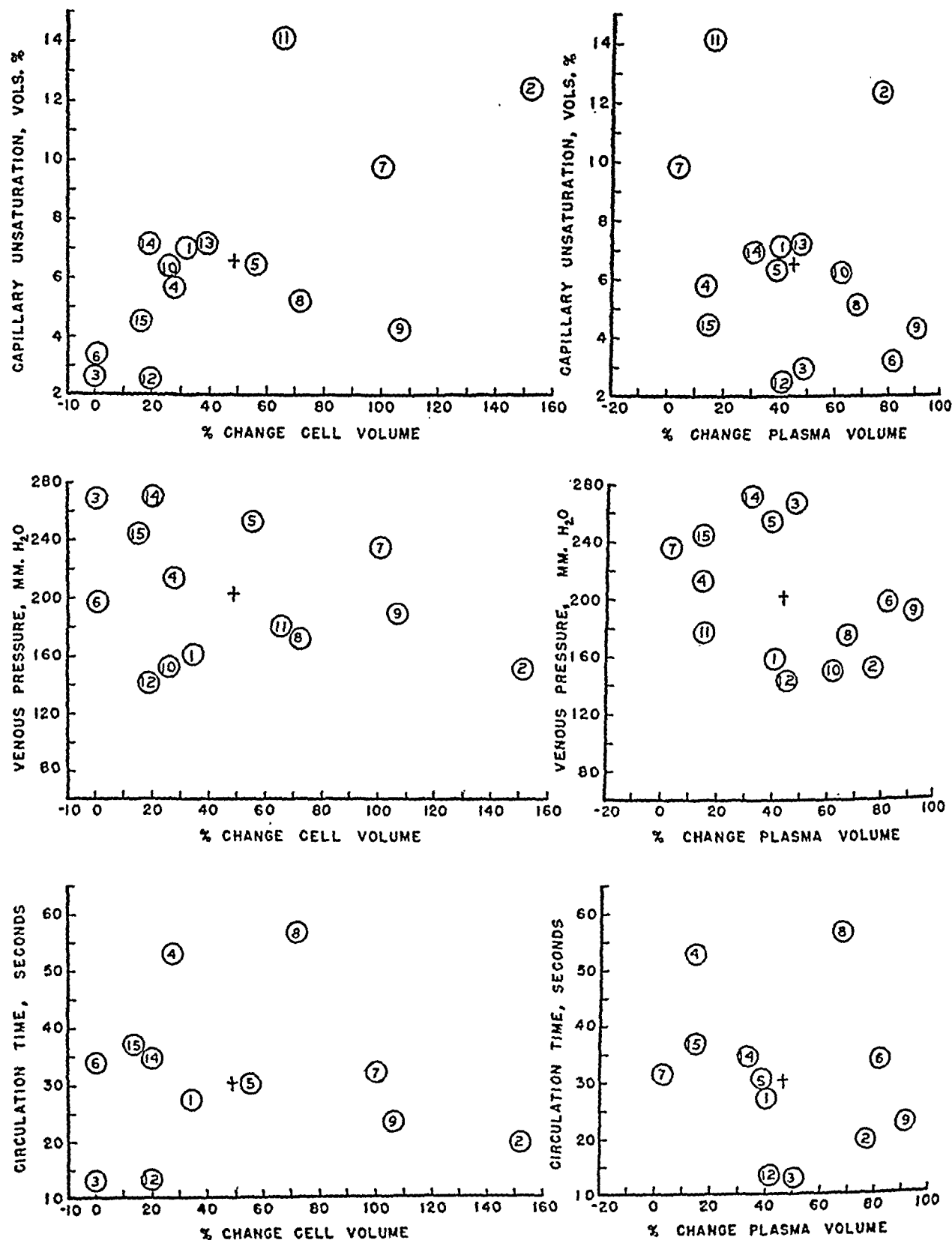


FIG. 1. CORRELATIONS BETWEEN CAPILLARY UNSATURATION, VENOUS PRESSURE, AND CIRCULATION TIME, AND THE PER CENT CHANGE IN THE BLOOD VOLUME AND ITS COMPONENTS IN 15 PATIENTS WITH SEVERE CONGESTIVE HEART FAILURE

# VOLUME OF THE BLOOD IN CONGESTIVE HEART FAILURE



FIG. 2. CORRELATIONS BETWEEN VARIOUS CARDIOVASCULAR MEASUREMENTS IN 15 PATIENTS WITH SEVERE CONGESTIVE HEART FAILURE

circulation time was compared with the blood volume, plasma volume, and cell volume (Figures 1 and 2 and Table III). There is no simple relation, the coefficients of correlation all being non-significant. As in the case of the venous pressure,

the interpretation must be strict: within a group of patients with severe heart failure, there is no simple relation between the blood volume or its fractions and the arm to tongue circulation time. Because both the venous pressure and the cir-

ulation time usually rise as congestive heart failure develops, it might be anticipated that there would be a simple relationship between these two. Within this group, such is not the case. There is no significant correlation between the venous pres-

TABLE III  
*Correlation between various measurements*  
Coefficients italicized are significant

Correlation	Cases	Coefficient	Significance (P=)
Venous pressure—blood volume	14	-0.418	no
Venous pressure—plasma volume	14	-0.400	no
Venous pressure—cell volume	14	-0.545	less than 0.05
Circulation time—blood volume	12	-0.105	no
Circulation time—plasma volume	12	-0.199	no
Circulation time—cell volume	12	+0.231	no
Capillary unsaturation—blood volume	15	+0.264	no
Capillary unsaturation—plasma volume	15	-0.257	no
Capillary unsaturation—cell volume	15	+0.630	less than 0.02
Venous pressure—circulation time	12	+0.099	no
Arterial CO <sub>2</sub> content—arterial O <sub>2</sub> saturation	15	-0.886	less than 0.01
Log circulation time— T. D. Heart	8	+0.929	less than 0.01
I. D. Chest			

sure and the circulation time, another statement which must be interpreted strictly as applying to this group of patients.

The saturation of arterial blood with oxygen ranged from normal levels to the low level of 41.8 per cent. The majority of the patients, eleven in number, show values between 80 and 94 per cent saturation, which is the common range in heart failure. Only four patients show values above 90 per cent, indicating that most of these patients have anoxic anoxia in varying degree.

While venous blood from the antecubital vein does not represent true mixed venous blood, it is, when taken under standard conditions, a useful index. The venous oxygen saturations observed range from 75 per cent to the very low value of 16.2 per cent. This lowest venous oxygen saturation occurred in the individual (number 14) who had the highest arterial saturation, representing purely stagnant anoxia. However, for the most part, the subjects studied showed mixtures of the stagnant and the anoxic forms of anoxia.

The best formulation of the degree of anoxia available from the data obtained is the capillary unsaturation expressed by Lundsgaard (8), as mentioned above. The mean capillary unsaturation in this group was  $6.6 \pm 3.4$  volumes per cent. Normal capillary unsaturation is 3.5 volumes per cent, and cyanosis appears at the level of 6.0.

To determine the relation of anoxia to the blood volume, the capillary unsaturation was compared with the blood volume and its fractions. There was no significant correlation with total blood volume nor with plasma volume, but there was a highly significant correlation with cell volume. The coefficient of correlation between the cell volume and the capillary unsaturation was +0.630.<sup>3</sup>

The carbon dioxide content of the arterial blood covers a wide range, from marked degrees of retention to quite low values. Taking 42 to 54 volumes per cent as the normal range (10), there are five cases below this range and two above it. The patients with marked carbon dioxide retention (numbers 2 and 11) had associated, marked anoxia. Both these patients had primarily pulmonary disease.

The vital capacity in all cases was low, ranging from 2.5 liters to 0.7 liters. The patient with the highest vital capacity (number 14) had also the highest oxygen saturation, and the individual with the lowest (number 2) had the lowest arterial oxygen saturation, but among the others there is no relationship apparent.

In those patients where roentgenograms of the chest were taken, measurement of the heart showed that all had large hearts. In order to allow for variation in stature of the individuals, the ratio of transverse heart diameter to internal thoracic diameter is used rather than the size of the heart itself. In normal patients, measured as in this study, this ratio is usually well below 0.5. It ranged in eight available cases between 0.564 and 0.750 with a mean of  $0.66 \pm 0.07$ .<sup>2</sup> An interesting relation obtains between the T.D./I.D. ratio and the circulation time. A linear plot of pairs of data suggested an exponential relation and a highly significant correlation was found between the logarithm of the circulation time and the T.D./I.D. ratio with a coefficient of correlation of 0.929.<sup>4</sup>

The hemoglobin concentration and the hematocrit give very little information with regard to

<sup>3</sup> A coefficient of correlation greater than 0.592 will occur in only two per cent of cases by chance with fifteen pairs of data. Fisher's Table V.A (11).

<sup>4</sup> A coefficient of correlation greater than 0.834 will occur by chance in only one per cent of cases with nine pairs of data. Fisher (11).

# VOLUME OF THE BLOOD IN CONGESTIVE HEART FAILURE

the actual volume of circulating blood or the absolute increase of either fraction. While the lowest values observed for volume of packed red blood cells occurs in those patients with the smallest change of total cell volume from normal, and while those patients with the largest volume of packed red cells had substantial increases, yet the correlation is very loose. Between the values of 40 and 45 volumes per cent of packed red cells there are five cases with red cell volumes ranging from normal to 106 per cent above predicted.

The plasma carbon dioxide combining power observed in these patients varies over a wide range from 33 volumes per cent to 76 volumes per cent. It is closely related to the arterial carbon dioxide content. There is no relation between the carbon dioxide content and the blood volume or its fractions within this group of patients.

Serum proteins averaged  $5.91 \pm 0.47^2$  grams per cent, in the lower range, but not by any means in the range of edema production. The volume of the blood and its fractions bore no simple relation to the plasma proteins.

Three patients were studied after improvement of their condition had been observed clinically. Patient number 12 had little anoxia and very little increase in cell volume. As she improved, there was diminution of her blood volume, due entirely to reduction in plasma volume. In the other two, the blood volume also diminished but the cell volume shared in the reduction, although not to the same extent as the plasma volume.

Patient number 14 who became worse when therapy was withdrawn and salt and fluids given freely, showed essentially the opposite change. Blood volume increased due to an increase in both fractions but principally due to plasma increase. Hemodilution occurred. Capillary unsaturation doubled due to stagnation, although changes in this value had not been striking in the patients who improved.

## DISCUSSION

The blood volume method described by Gregerson, Gibson, and Stead (12), using the blue dye T-1824 suggested by Dawson, Evans and Whipple (13), was adapted to clinical use by Gibson and Evans (3). It is thought to be more accurate than previous dye methods because of improved colorimetry, correction for hemolysis, allowance

of adequate time for mixing, and correction for the disappearance of dye from the blood stream. Gibson and Evans found two-thirds of their normal patients had blood volumes within plus and minus 10 per cent of the average for their sex, and that predictions of greater accuracy could be made on the basis of sex and height (or surface area).

Freeman, Freedman, and Miller (14) found that under certain conditions, notably shock, the blue dye may escape from the blood stream, as evidenced by failure of the extrapolated dye line to check with subsequent determinations, steep slope of subsequent dye lines, and the presence of dye stained fluid in the tissue spaces, cisterna chyli, pericardial sac, and the lumen of the intestine. In the cases studied, there is no reason to believe that permeability of the vascular tree to the dye existed, because there was no unusual dye line slope and blue staining of transudates was never observed although many were examined. We concluded that determination of the plasma volume in these patients was as accurate as in Gibson and Evans' standard series, that is, better than 10 per cent in two-thirds of the cases studied.

More serious objection exists to the determination of the cell volume. Smith, Arnold, and Whipple (15) ascribed the lack of agreement between blood volume methods which depend on plasma volume measurements and those which depend on cell volume measurements to variation in the ratio of cells to plasma in different parts of the vascular tree. Ebert and Stead (16), by their bleeding experiments, have recently produced evidence that this is indeed the case as has Hahn (17), using radioactive red cells. It is evident, then, that in the absolute values reported for red cell volume, there is an error of unknown magnitude which arises from the assumption that the hematocrit ratios obtained with venous blood represent the average body hematocrit. However, the relative changes in the total cell volume probably are significant.

The blood volumes found in the nineteen cases studied were all above normal, and in 13 cases, the increase was over 20 per cent. This is in accord with the finding of Gibson and Evans (1, 2) that in congestive heart failure, patients with diminished blood volumes do not occur. It was

not found, however, that there was any significant difference in the change in the two fractions. Great variability exists in the elevation of the blood volume itself, and in the plasma and cell volumes in the individual cases, although, in the majority, elevation of both components occurred.

Consideration of changes observed in the volume of the blood may well involve separate consideration of the two fractions, for there is evidence that control of the watery and of the cellular fractions of the blood are mediated in different ways.

Anoxemia has been considered as a factor in changes in hemoglobin and red cells since the work of Bert in 1882 (18), and Wintrobe and Harrop (19) have reviewed the hematologic effects of anoxemia. In brief, there is an increase in the circulating red cell volume in chronic anoxemia due to altitude, with an increased total blood volume, due largely to increase in the cells. Upon return to normal levels, the cell volume falls and there is a slight compensatory rise in the plasma volume. Compensatory polycythemia is thus a cellular affair, with essentially normal plasma volume. For reasons not understood, anoxemia does not always produce a polycythemia as noted by Kaltreider, Hurtado, and Brooks (20) and Lemon (21).

In heart disease, however, the change in the blood volume involves both fractions. From the work of Bolton (22), it would appear that the earlier change is in the plasma volume. It might be supposed that anoxemia does not become a factor until stagnation and pulmonary congestion produce it, and in the train of anoxemia, increased hematopoietic activity ensues.

There is increased hematopoietic activity in congestive heart failure: Weil in 1901 (23) and Fromherz in 1903 (24) noted virtual replacement of the yellow marrow by red in polycythemic congenital cardiac patients. Waller and Blumgart (25) showed a definite reticulocytosis, diminished excretion of urobilin, and increased red cell mass before congestive heart failure becomes advanced. With onset of clinical improvement, when reduction of the total blood volume ensues, the red cells undergo hemolysis (due in part to the observed increased fragility), the level of bilirubin in the blood rises, the excretion of urobilinogen increases, and the reticulocyte percentage becomes smaller.

From these findings and the data reported

above, it appears that the cellular portion of the blood responds to the observed anoxemia in heart failure according to the degree in which it is present, due to increase or decrease in hematopoietic activity and changes in blood destruction as the patient becomes more or less anoxicemic. More satisfactory formulation of the degree of anoxemia than the "capillary unsaturation" should show a closer relation, for it is well recognized that it is the oxygen tension, not the concentration, which is of primary importance.

Nothing in the data accumulated bears a simple relation to the plasma volume. It does not correlate with circulation time, anoxemia, plasma carbon dioxide combining power, nor plasma proteins. It is more labile than the cell volume as evidenced by the four repeated studies, a phenomenon observed by Gibson and Evans (1) who noted the more rapid decline of the plasma volume with clinical improvement, but also a subsequent cell volume decline.

The negative correlation observed between the venous pressure and the cell volume within this group of patients is difficult to understand. Survey of a group of patients such as Gibson's (1) shows general rise of the blood volume and its fractions with rising venous pressures. Despite the statistical indication of probable significance, it is most likely an artificial result of selection of these patients, who all had elevated venous pressures; for in other factors studied, a better distribution over the range from normal to marked abnormality was obtained. Conceivable, however, is the hypothesis that if the elevation of venous pressure is compensatory, then in those who respond less well, more anoxemia occurs and hence greater hematopoietic stimulation. In the interpretation of these results, it is important to remember that these correlations were derived from a very limited series of patients, suffering from severe congestive heart failure. It would appear that the control of the plasma volume in severe congested cardiacs is mediated by mechanisms not studied in this group of patients.

The role of the blood volume in the complete picture of congestive heart failure remains obscure. From the known response of the cell volume and the hematopoietic system as a whole to anoxemia, and from the observed parallelism of increasing cell volume with increasing grades of anoxe-

mia, the contribution of the cell volume to the increased blood volume of congestive heart failure is comprehensible. It appears likely, however, that the changes in the plasma volume are primary to the changes in cell volume, because the plasma volume is more labile, because heart failure is at least moderately advanced before anoxemia of any significant degree obtains, and because rise of the plasma volume occurs first in experimental congestion. The data obtained and the analyses made do not shed light upon the question of whether the increased blood volume is the result of the increase in venous pressure or the cause of it.

Failure of the venous pressure to correlate with the circulation time is at variance with the general experience that as heart failure advances, the circulation time and the venous pressure both increase. Two factors appear to contribute to the observations made here: first, the selection only of severely congested cardiacs, and second, the impossibility of evaluation of right versus left heart failure from the data obtained.

Most interesting is the relation between the circulation time and the transverse diameter of the heart. First mention of this relation appears in the French literature in the work of David and Bouvrain (26). Since there are only nine cases observed in this study, it is not advisable to speculate upon this relationship until further study adds a larger amount of data.

The plasma protein values found agree with the generally accepted view that in heart failure, the proteins are lower than normal, but not lowered to a degree sufficient to produce edema. Interpretation of the significance of plasma proteins in heart failure should only be made in the light of the state of fluid balance of the patient, according to Stewart (27), and as these patients were not followed in this regard, no comment should be made upon the proteins.

#### SUMMARY AND CONCLUSIONS

1. In fifteen patients with severe congestive heart failure, the blood volume, arterial and venous blood pressure, arm to tongue circulation time, vital capacity, arterial and venous oxygen and carbon dioxide content and oxygen combining power, volume of packed red cells, plasma carbon

dioxide combining power, and plasma total proteins were measured and are reported.

2. The blood volume was increased in all cases, and in most, the increase was substantial.

3. There was great variability in the increase and in the degree to which the plasma and the cells contributed to the increase, and no statistically significant difference was observed between the increase in cell and plasma volume.

4. There was no simple correlation between the total blood volume and the other measurements made, nor between the venous pressure and the circulation time.

5. A negative correlation of moderate significance was found between the venous pressure and the plasma volume.

6. The logarithm of the circulation time bore a linear relation to the ratio between the transverse diameter of the heart and the internal diameter of the chest in eight cases where roentgenograms of the chest were taken.

7. The venous hematocrit and hemoglobin gave little indication of the actual volume of circulating cells.

8. There was a highly significant correlation between the degree of anoxemia measured by Lunds-gaard's "capillary unsaturation" and the increase in cell volume.

9. In three patients who improved, all measurements tended towards normal, the plasma volume decreasing more than the cell volume. In the patient who grew worse, the reverse was the case.

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# QUANTITATIVE RELATIONSHIP BETWEEN BASAL METABOLIC RATE AND THYROID DOSAGE IN PATIENTS WITH TRUE MYXEDEMA

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Patients with myxedema differ from other subjects in their response to oral thyroid extract (1). The precise nature of this difference has not, however, been clearly defined. The present study originated in the observation that certain non-myxedematous subjects could tolerate very large daily doses of oral thyroid extract without demonstrable effect on the basal metabolic rate. This behavior, so different from the acute sensitivity of patients with true myxedema even to small amounts of thyroid extract, demanded further investigation. As a preliminary step, the present paper supplements the work of Means and Lerman (2) in defining as exactly as possible the relationship between thyroid dosage and metabolic response in patients with true myxedema.

## MATERIAL AND METHODS

*Clinical diagnostic criteria.* Twenty-eight patients with fully developed myxedema were included. The difficulties in some cases of making a positive diagnosis were fully recognized, and all questionable cases were rejected. In fifteen cases, the clinical diagnosis was confirmed by the demonstration of a high serum cholesterol, by a low blood or serum iodine, or by both.<sup>2</sup> The remaining patients had been seen before the introduction of these techniques of blood chemistry, or refused to discontinue thyroid medication, so that the diagnosis rested on clinical grounds. These included, in every case, the initial presence of the disease and the prompt and complete return of the patient to a normal state with daily administration of one or two grains of thyroid extract. In no case was an initially low basal metabolic rate alone considered evidence of myxedema. Subjective improvement on thyroid medication without striking objective evidence of physical change was also excluded

<sup>1</sup> Some of the data upon which this paper is based were included in a thesis submitted by Joseph Criscuolo to the Yale University School of Medicine, in partial fulfillment of the requirements for the degree of Doctor of Medicine, 1938.

<sup>2</sup> We are indebted to Dr. Evelyn Man and to Dr. Douglas Riggs for these chemical data.

from consideration. In several instances, the diagnosis was proven by withholding thyroid until definite evidence of myxedema manifested itself. The time necessary for this to develop varied considerably from patient to patient, but was not less than two weeks nor more than eight weeks. Two cases with total thyroidectomy for heart disease were included. Subjects with hypometabolism following subtotal thyroidectomy were not in general included, since the thyroid deficiency in these patients is apt to be transitory or at least incomplete (3, 4). The only exception was a single subject, followed for many years, who developed full-blown myxedema whenever thyroid extract was discontinued.

*Technique of study.* The basal metabolic rate (which will hereafter be referred to as the "BMR") of each patient was determined from time to time over a period of months or years. The BMR was then correlated with the dose of dried thyroid which the patient had been receiving during the preceding month. Tablets of U. S. P. dried thyroid were used. The patients were usually ambulatory, although a few observations on hospitalized patients are included. The BMR was determined with a Benedict-Roth machine in the usual fashion, in the morning, after thirty to sixty minutes of complete physical rest. Patients were all in the postabsorptive state. Two curves were taken, and if they did not check within five per cent, a third curve was obtained. BMR determinations in the same patient done on successive days usually checked within less than ten per cent.

*Mode of analysis.* BMR determinations were included only if the patient had been on the same daily dose of thyroid for at least one month before the metabolism test. The patient was regularly questioned at the time of the test, and the determination was excluded if there had been any irregularity of dosage. No subjects known to be habitually careless about taking medication were included. All acceptable values of the BMR in the same subject corresponding to each level of dosage were then collected, and an average taken. These averages are included in Table I; the small figures beside each average indicate the number of individual BMR determinations upon which the average was based. The average deviation of the mean is also included in all instances in which five or more determinations were available. An exception to this procedure was made in the selection of the BMR associated with no administration of thyroid. Here, the lowest rather than the average value was taken, since at most, only two or three determinations at this level



TABLE I  
Basal metabolic rate and daily thyroid dosage

Case	Average BMR while taking oral thyroid daily* Dosage, grains per day												
	0†	0.25	0.50	0.75	1.00	1.25	1.50	1.75	2.00	2.25	2.50	2.75	3.00
1	-35		-23 <sub>3</sub>		-21 <sub>6</sub> ±11		+1 <sub>1</sub>		-10 <sub>8</sub> ±5				
2	-41				-4 <sub>1</sub>		-5 <sub>9</sub> ±2		+4 <sub>8</sub> ±6				
3	-34			-24 <sub>1</sub>	-10 <sub>1</sub>		-16 <sub>20</sub> ±6		+4 <sub>1</sub>	-5 <sub>7</sub> ±4	-5 <sub>3</sub>		
4	-30								-11 <sub>9</sub> ±3		+1 <sub>4</sub>		-9 <sub>7</sub> ±5
5	-38				-4 <sub>3</sub>				-4 <sub>2</sub>				-3 <sub>1</sub>
6	-38								-4 <sub>10</sub> ±7				-11 <sub>18</sub> ±5
7	-42		-33 <sub>1</sub>	-27 <sub>1</sub>	-25 <sub>1</sub>				-15 <sub>1</sub>				-9 <sub>2</sub>
8	-31				-11 <sub>1</sub>				+14 <sub>1</sub>				
9	-30								-3 <sub>2</sub>				+13 <sub>1</sub>
10	-34				-3 <sub>7</sub> ±3				+8 <sub>1</sub>				
11					-16 <sub>2</sub>		-12 <sub>1</sub>		-5 <sub>2</sub>				
12	-31		-16 <sub>2</sub>		+5 <sub>1</sub>								
13†	(-22)§	-11 <sub>4</sub>											
14	(-18)§				+7 <sub>9</sub> ±5								
15	(-26)§	-24 <sub>1</sub>			-6 <sub>3</sub>								
16	-31								-13 <sub>1</sub>				
17	-43								-20 <sub>2</sub>				
18	-39								-9 <sub>2</sub>				
19	-27						+11 <sub>4</sub>						
20	-24								-4 <sub>3</sub>				
21									-9 <sub>1</sub>				
22									-3 <sub>2</sub>				
23	-38				-19 <sub>1</sub>		-26 <sub>1</sub>						-20 <sub>8</sub> ±5
24	-41				-15 <sub>2</sub>								
25	-34				-14 <sub>2</sub>		-11 <sub>1</sub>						
26	-48				-24 <sub>1</sub>		-14 <sub>1</sub>		-11 <sub>1</sub>				
27	-19								-11 <sub>1</sub>				
28	-28								-4 <sub>9</sub> ±4		-3 <sub>4</sub>		+2 <sub>2</sub>

\* Large figures represent average BMR's; small figures represent number of individual BMR determinations.

† Minimum BMR.

‡ BMR on 0.125 grains of thyroid daily was -15 per cent.

§ Lowest BMR following total thyroidectomy; true myxedema not allowed to develop.

were available, and since it was felt that a minimum figure would be less misleading than an average one.

#### GENERAL RESULTS

The averages of Table I, calculated in the manner which has just been described, indicate at once two general facts. (1) The dose necessary to restore the BMR to normal varied from one to three grains of thyroid daily. In a few patients, one grain daily sufficed; the majority required about one and one-half or two grains, while some at times required as much as three grains daily. In no subject, with the possible exception of case 23, was there any indication that more than three grains daily were necessary. Patients requiring only one or one and a half grains daily usually became nervous and ill if given more (case 10, for example). The requirements for thyroid were apt to be somewhat greater in heavy than in slight subjects, but this correlation was very irregular. (2)

There is no evidence of any failure of response to thyroid. In every instance, the BMR during thyroid medication remained consistently greater than the BMR while no medication was being given.

#### QUANTITATIVE RELATIONSHIPS

In all cases, there was a positive correlation between the height of the BMR and the current dose of thyroid. On a statistical basis, this is apparent in Figure 1, in which all values of Table I are represented by points. The distribution of points is similar to that observed by Means and Lerman in their study of myxedema (2). The plot of Figure 1 is, however, misleading, in that it suggests that the first increments of thyroid substance are much more effective in raising the BMR than are subsequent increments. This conclusion is not warranted, since all patients were not tried out on all levels of dosage. As a rule, each patient was

given just enough thyroid to bring the BMR back to normal. This might vary from one to three grains daily, and might vary from time to time in the same patient. Patients requiring only one grain daily to restore their BMR to normal were never given two or three grains daily, while, on the other hand, only those patients requiring the full three grains daily to restore the BMR to normal are included in the three grain column. Had all patients been given three grains daily, it seems

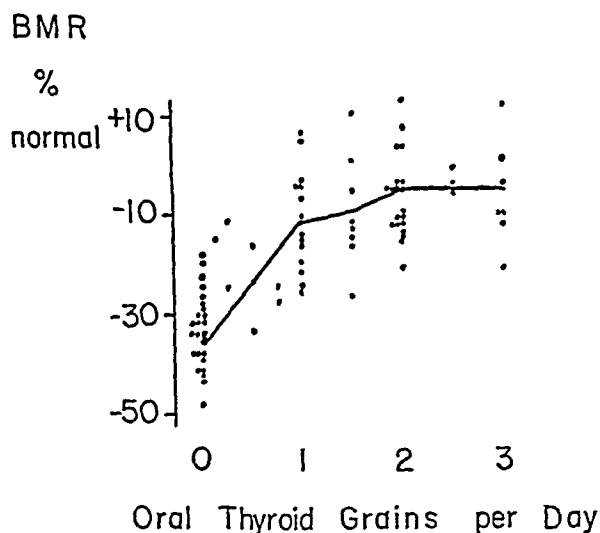


FIG. 1. STATISTICAL RELATIONSHIP BETWEEN BMR AND DOSAGE OF ORAL THYROID IN THE TWENTY-EIGHT CASES OF TABLE I

The heavy line connects the average values for the BMR at each level of thyroid dosage.

most probable, on the basis of acute experiments, that a number of supranormal values would have been found in the three grain column, with a corresponding increase in the average BMR. The apparent evidence for "diminishing return" from thyroid medication has, therefore, little validity, since it may only represent the artificial conditions under which these averages were obtained.

In principle, a more satisfactory approach to this matter might be made by following the BMR in the same individual at different levels of dosage. There is evidently a distinct linearity in many cases, such as 1, 2, 8, 9, 10, and 11, while in several others, such as 3, 4, 5, and 6, the curve unmistakably flattens out as higher levels are reached. If we confine our attention to the effects of daily dosages of one and of two grains, respectively, in

the same individual, the variability is equally evident. Of the eight subjects so studied, four showed as much increment of BMR from the second grain as from the first, while four showed a smaller increment from the second grain.

Here too, however, the manner in which the thyroid was administered must be considered before the results can be interpreted. In all cases with flattening of the curve at higher levels of dosage, the patients were not tested impartially at various levels of dosage. Instead, the dose was usually increased or decreased in order to keep the level of the BMR constant. For example, in cases 4 and 6, followed for many years, it was customary to raise the dose to three grains daily whenever the BMR fell a little or the patient gained a little weight, and to reduce it again to two grains daily whenever the BMR rose a bit or the patient became nervous. Indeed, in case 6, the policy of raising the dose to three grains whenever the BMR tended spontaneously to be lower resulted in a lower average BMR while on three than while on two grains daily! The general result was, of course, to keep the BMR about the same regardless of dose. Attempts were made in several instances deliberately to raise the thyroid dosage above the amount required to restore the BMR to normal, but usually the patients refused to continue the increased dosage for any length of time, because of nervousness, tachycardia, or weight loss. In case 1, for example, thyroid dosage was experimentally varied between one and two grains daily, regardless of the previous BMR or the patient's condition. Omitting the elevated value on one and a half grains daily, which was based on one BMR determination only, the line between the levels at zero, one, and two grains is entirely straight.

#### VARIABILITY OF THE BMR IN THE SAME SUBJECT

It has already been pointed out that the same patient may require more or less thyroid at different times in order to maintain the same level of the BMR. This means that the BMR in the myxedematous, and supposedly thyroidless, individual may vary spontaneously from time to time. Patients differ with respect to the degree of variability of the BMR. Thus, in case 23, the BMR on three grains daily varied between -9 per cent and -29 per cent, and in case 3, the BMR on one and

a half grains daily was at one time as low as — 28 per cent and at another as high as + 2 per cent. This is a wide range of variability; in most subjects, the fluctuations were somewhat less extreme. Often, however, the variation exceeded that to be expected from the technical uncertainties of BMR determination.

#### ABSENCE OF TOLERANCE TO THYROID EXTRACT

The variations which have just been described seemed to be cyclical rather than progressive, and in the same patient, the BMR on a given dose might fluctuate over a period of years without any long term trend. No tendency toward "tolerance" to larger and larger doses of dried thyroid, such as has occasionally been observed in non-myxedematous subjects, could be detected. This is clearly brought out in Table II. The dose of thyroid

TABLE II

*Absence of tolerance to oral thyroid in patients with myxedema, followed for a number of years*

Case	Years followed	Daily dose of thyroid necessary to maintain normal BMR	
		When first seen	When last seen
1	19	3	2 to 3
2	14	3	2 to 2.5
3	8	1.5 to 2	2.5
4	6	3	3
5	5	1	2
6	6	2	2
7	5	2	2
8	4	1	1.5

necessary to maintain a normal BMR did not change significantly in any of these patients over periods ranging from four to nineteen years.

#### COMPARISON OF THE EFFECTS OF ORAL THYROID AND INTRAVENOUS THYROXINE

Data bearing on this point are available in two cases. In one case, 0.2 mgm. of thyroxine daily for three months restored the BMR to — 14 per cent, a level which could subsequently be maintained with one grain of dried thyroid daily. In the other case, 0.6 mgm. of thyroxine daily for six weeks restored the BMR to + 1 per cent, a level which at that time could be maintained with a daily dose of three grains of oral dried thyroid daily.

#### DISCUSSION

The myxedematous subject certainly does not react to thyroid medication with the mathematical predictability of a machine. Some individuals require a larger dose than others, both absolutely and in proportion to their body weight. Factors other than thyroid medication obviously affect the BMR, since this may vary from time to time without change in the dose of thyroid. Nevertheless, beyond rather narrow limits the myxedematous patient appears to be at the mercy of his thyroid medication. If this is lowered below a certain point, he will always develop evidences of deficiency; if it is raised above a certain point, he will develop signs of toxicity. The intermediate range of dosage is not great, the leeway being not more than a grain or so daily. He apparently never develops a tolerance to dried thyroid, and never requires more than about three grains a day for full maintenance.

#### CONCLUSIONS

(1) Myxedematous patients do not develop any tolerance to dried thyroid, even after years of medication.

(2) In myxedema, the dose of dried thyroid necessary to restore the basal metabolic rate to normal regularly lies between one and three grains daily. It may, however, vary somewhat in the same individual from time to time.

(3) The relationship between BMR and the dose of dried thyroid tends to be linear, at least while the BMR is subnormal.

(4) The quantitative response of the myxedematous subject to dried thyroid is specific and reproducible. The diagnosis of myxedema may properly be questioned if this characteristic response cannot be demonstrated.

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# TOLERANCE TO ORAL THYROID AND REACTION TO INTRAVENOUS THYROXINE IN SUBJECTS WITHOUT MYXEDEMA

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The failure of certain non-myxedematous subjects to respond to large doses of oral dried thyroid has been previously noted (1). The lack of responsiveness contrasts sharply with the sensitivity to dried thyroid invariably evinced by patients with true myxedema (1, 2). In this paper, the responses of various non-myxedematous subjects to thyroid medication are described. In certain of these individuals, the response to intravenous thyroxine was compared with that to oral thyroid substance.

## MATERIALS AND METHODS

The mode of attack paralleled that previously employed in the study of myxedematous patients (1). Over a period of months or years, the basal metabolic rate (hereafter referred to as the "BMR") of each patient was determined at intervals. Oral dried thyroid in various dosages, or courses of intravenous thyroxine, were given from time to time during the period of study. The response of the patient to each procedure could thus be evaluated against the background of his entire course. Difficulties in interpretation arising from shifts in the level of the BMR, from changing responses to the same dose of thyroid and from single aberrant determinations,

were in this way minimized. No subjects who did not take the same dose of dried thyroid for at least two months were included. Special care was taken to eliminate all patients thought to be unreliable in taking their medication. All subjects with a clinical picture suggesting the presence of myxedema were excluded. About thirty-six subjects in all were selected, from a much larger group of patients studied, for inclusion in this report; five of the patients were studied in great detail. Six patients received one or more courses of intravenous thyroxine (Squibb), injections being given every three or four days. The dry thyroxine was dissolved in dilute sodium hydroxide a few minutes before injection. The method for the determination of serum iodine was that of Riggs and Man (3).

## RESULTS

(A) *Lack of effect on the BMR of long continued administration of dried thyroid*

(1) Subjects taking five or more grains of thyroid extract daily without elevation of the BMR  
(A44014, A13481, 85533)

In Figures 1, 2, and 3 are presented graphically the courses of three patients who repeatedly exhibited an ability to tolerate doses of thyroid of

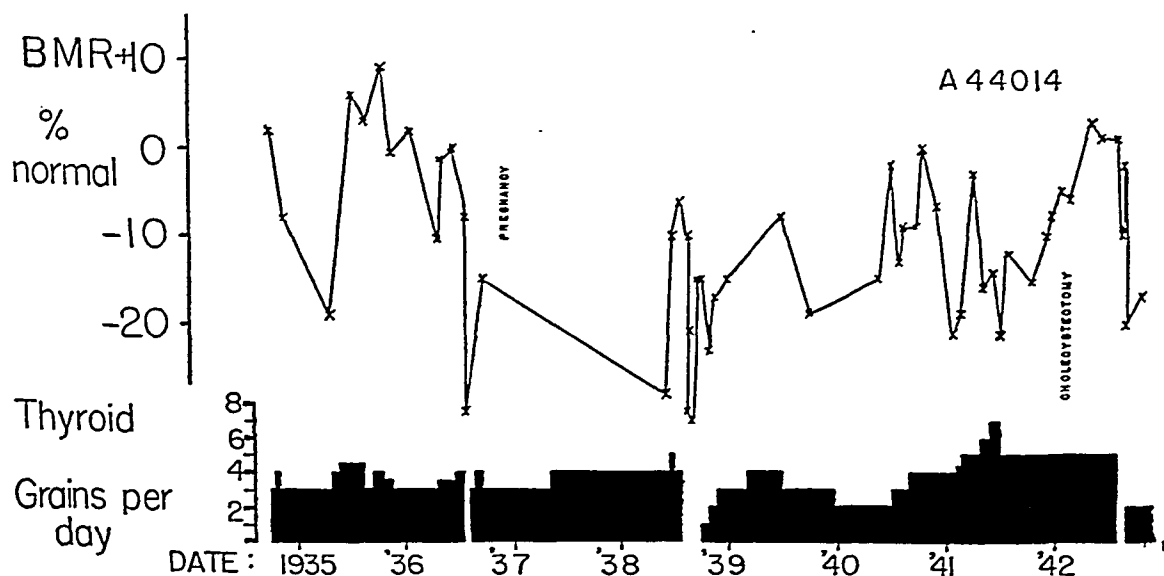


FIG. 1. NON-MYXEDEMATOUS SUBJECT WITH PERSISTENTLY LOW BASAL METABOLIC RATES, EVEN WHEN TAKING FIVE OR MORE GRAINS OF THYROID BY MOUTH DAILY

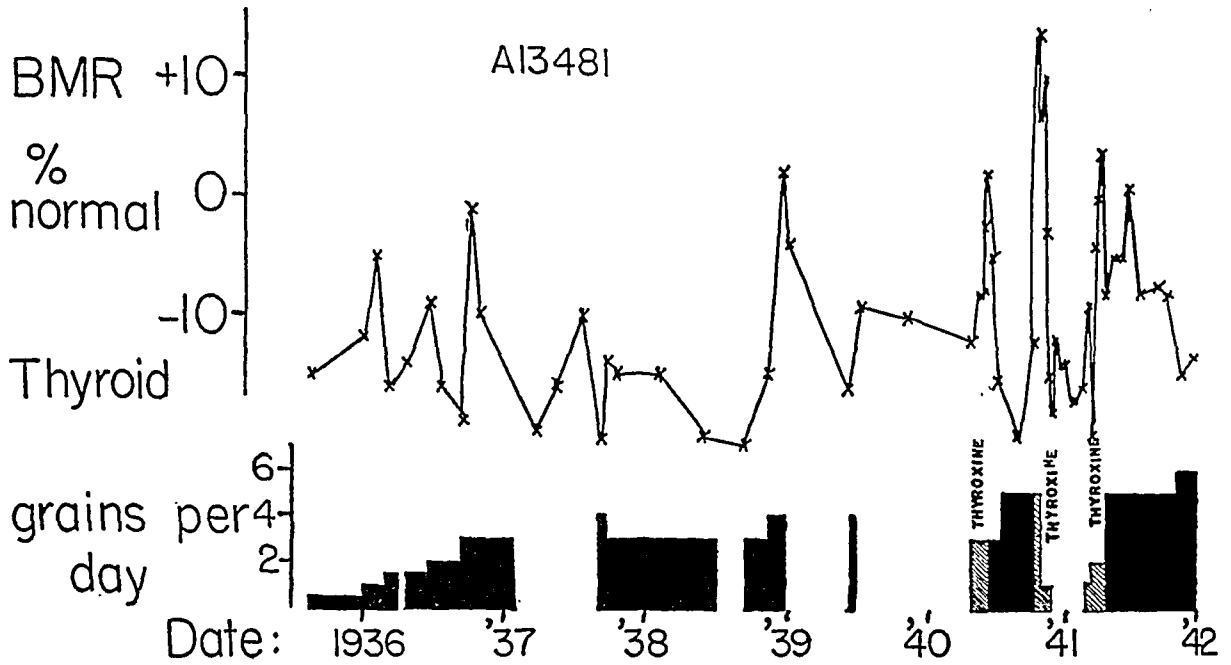


FIG. 2. NON-MYXEDEMATOUS SUBJECT WITH PERSISTENTLY LOW BASAL METABOLIC RATES, EVEN WHEN TAKING FIVE OR MORE GRAINS OF THYROID BY MOUTH DAILY  
Note response to intravenous thyroxine in iodoequivalent amounts.

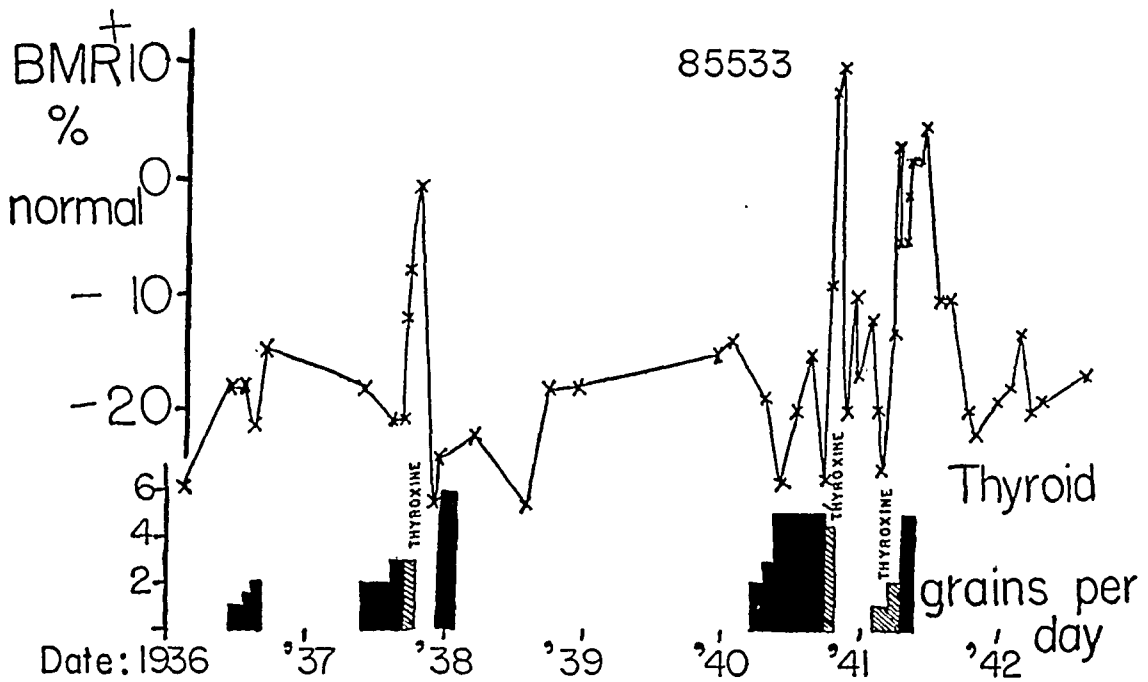


FIG. 3. NON-MYXEDEMATOUS SUBJECT WITH PERSISTENTLY LOW BASAL METABOLIC RATES, EVEN WHEN TAKING FIVE OR MORE GRAINS OF THYROID BY MOUTH DAILY  
Note response to intravenous thyroxine in iodoequivalent amounts.

five or more grains without any persistent increase of the BMR or of the pulse rate. It cannot be said, however, that the thyroid extract was totally without effect in these subjects. For example, case A44014 (Figure 1) received thyroid medication for eight years, with only three brief interludes, in doses ranging between three and seven grains daily. There seems to have been a gradual

trend toward a lower level of metabolism as the years have gone by. Although on one occasion she managed to tolerate seven grains of thyroid daily without subjective disturbance and with a BMR of  $-21$  per cent, at other times the administration of five grains daily sufficed to maintain the BMR at normal levels. Furthermore, the BMR of this patient always dropped below its

current level with each interruption of thyroid medication, though it later tended to rise spontaneously again. There was also a very rapid weight gain whenever thyroid medication was interrupted, together with a subjective sensation of weakness. The weight gain and the subjective weakness appeared within two or three days after the thyroid was stopped, so that they could hardly have been due to complete exhaustion of thyroid effect. Serum iodine concentration was also affected by thyroid dosage (see Table IV, below). Patient A13481 (Figure 2) exhibited perhaps the most complete indifference to thyroid medication, since the BMR did not rise with as much as six grains daily, and did not fall even when thyroid medication was stopped. Pulse rate was similarly unaffected. There were, however, two instances, one in 1938 and the other in 1941, when the BMR did increase temporarily when the thyroid dosage was increased. Also, there was a marked weight gain whenever thyroid was stopped, indicating that it was not wholly without metabolic effect. Patient 85533 (Figure 3) was similarly unaffected by a dose of five grains daily, save for a slight tendency to gain weight whenever dried thyroid was discontinued.

In contrast to this indifference to oral thyroid, cases A13481 and 85533 reacted markedly to intravenous thyroxine (A44014 never had a trial of thyroxine). BMR and pulse rose, weight fell,

and nervousness and tachycardia developed. The quantitative aspects of this response to thyroxine will be considered below (see Table III).

- (2) Subjects without response of the BMR to a daily dosage of three or four grains, but with response to five or six grains of dried thyroid daily (16186, A29887)

Patient 16186, whose course is presented in Figure 4, had a BMR of about  $-20$  per cent whenever she received no thyroid. On three grains daily, there was no consistent change; on four grains daily, the BMR was sometimes but not always a little elevated; while on five grains, the BMR regularly rose to the average normal level. This increase was accompanied by a slight rise in the pulse rate, but there was no real tendency toward weight loss. Iodine concentration in serum also responded to changes in thyroid medication (see Table IV).

The response of patient A29887 was quite similar to that of 16186. The reaction of the BMR during the eight months of administration of three grains daily is especially interesting, in that an initial elevation of the BMR to  $+20$  per cent was succeeded by a fall almost to the initial level, in spite of the continued administration of dried thyroid. A daily dose of four grains was also without much effect, but six grains daily produced an elevation of the BMR to  $+25$  per cent,

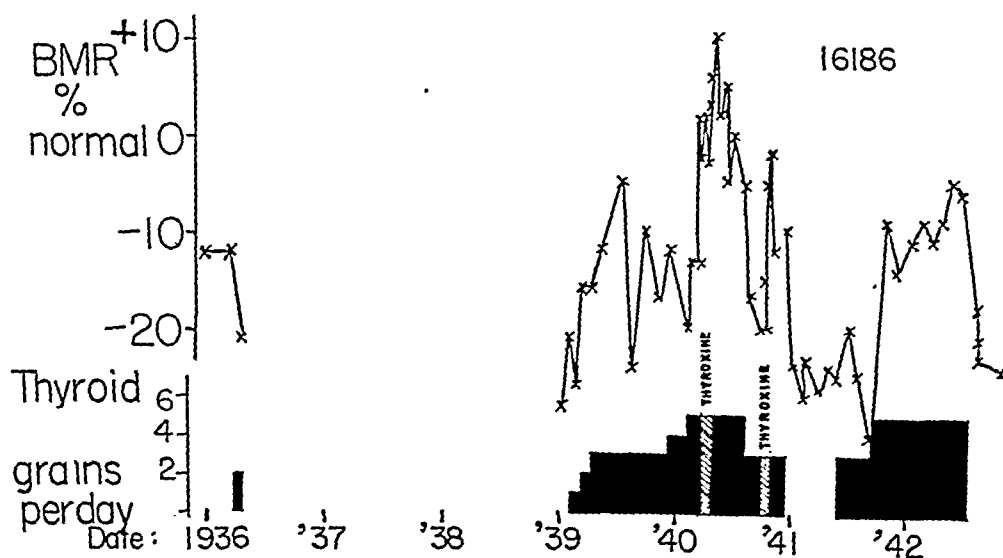


FIG. 4. RISE OF THE BMR WITH FIVE BUT NOT WITH THREE GRAINS OF DRIED THYROID DAILY; POSITIVE RESPONSE TO INTRAVENOUS THYROID IN IODOEQUIVALENT AMOUNTS

TABLE I

*Patients, other than those of Figures 1 to 5, failing to exhibit any consistent elevation of the BMR after taking thyroid extract for several months*

Number	Age	Diagnosis	Dose of thyroid,	Duration	Initial BMR	Final BMR
	years		grains per day	months	per cent normal	per cent normal
1	60	Arteriosclerosis, hypometabolism	1, 2, 3, 4	5	-18	-18
2	16	Obesity, hypometabolism	2, 3, 4	30	-15	-23, -6
3	55	Arteriosclerosis, hypometabolism	2, 5, 3, 4	7	-31†	-33
4	48	Neurosis, hypometabolism	1, 2	6	-20	-14
5	12	Alopecia areata	2, 3	48	-14	-8
6	55	Arteriosclerosis, hypometabolism	1, 2, 3	5	-29	-20
7	39	Hypometabolism	2, 5	5	-20	-15
8	27	Syphilis, hypometabolism	1, 2	9	-25	-23, -15
9	16	Obesity	1, 2	5	-16	-12
10	35	Hirsutism, obesity	1, 2	4	-11	-6
11	20	Infantilism, mild	1, 2	96	-16	-11
12	32	Obesity, neuralgia	1, 1.5	6	-19	-18
13	28	Diabetes, obesity	1	5	-12	-19
14	53	Simple goiter, hypometabolism	1	40	-24, -1	-14
15	43	Anemia, hypometabolism	1	17	-18	-17
16	37	Simple goiter, hypometabolism	0.5, 1	18	-25	-22
17	60	Simple goiter, subtotal thyroidectomy, hypertension	1	8	-19	-17
18	42	Simple goiter, subtotal thyroidectomy	1	17	-12	-19
19	35	Obesity	0.5, 1	8	-4	-18

\* All but number 19 were females.

† -6 per cent two years later.

which was sustained for the two-month period during which this large dosage was continued. There was a concomitant elevation of the pulse rate, weight loss, and slight nervousness. Subjective disturbance was, however, slight. Unlike the other four patients showing adaptation to large doses, this patient had initially a normal BMR.

Both of the patients in this group reacted with an elevation of the BMR, and an increased pulse rate to intravenous thyroxine. Their response to this medication will be considered in detail below (see Table III).

### (3) Adaptation to four grains or less of oral thyroid daily

In Table I are summarized the diagnoses and metabolic data concerning nineteen other subjects failing to exhibit any consistent elevation of the BMR after taking dried thyroid for several months. Necessarily, such a compact summary omits much pertinent detail. In many cases, it was difficult to assign a definite value to the control BMR, since the BMR on no thyroid medication varied considerably from time to time. The BMR values in the table are frequently averages of two or more determinations. Nevertheless, it is obvious that the BMR in these patients failed to exhibit much change, even though oral thyroid

extract had been given for weeks or months. In several of these subjects, there was a transitory elevation of the BMR after three or four weeks of thyroid medication, with a subsequent decline to initial levels. Occasionally, the BMR actually decreased.

This was not an unselected group of cases. In most of them, dried thyroid extract was given for its possible therapeutic effect on hypometabolism, on obesity, or on both. Since attempts to raise the dose of thyroid above one grain daily were only successful in those patients who did not react unfavorably, this list automatically excludes many of those who did not tolerate it well. Subjective reaction and metabolic response were not invariably linked. For example, case 19 developed palpitation and perspired intensely whenever the dose of thyroid was increased above one grain daily, yet the BMR on one grain was actually lower than the initial BMR.

All but one of the patients were females. This may be in part a reflection of the economic situation of the group studied, since the women, more readily than the men, could afford to spend regularly a portion of the working day waiting in the office or dispensary and having the repeated BMR determinations required by this study. There was but one patient who did not at one time or another

TABLE II

*Patients showing no tolerance of the BMR to prolonged administration of oral thyroid extract \**

Number	Age	Diagnosis	Dose of thyroid,	Duration	Initial BMR	Final BMR
	years		grains per day	months	per cent normal	per cent normal
1	60	Arteriosclerosis, hypometabolism	1, 2, 3	7	-24	-9
2	54	Simple goiter	0.5 to 2.5	10	-10	+9
3	41	Hypometabolism	1.5, 2	6	-18	-3
4	35	Hypometabolism	2	5	-12	+7
5	52	Hypometabolism	2	2	-23	-8
6	24	Obesity, headaches	1, 2	9	-15	-1
7	35	Obesity, hypometabolism	1, 2	8	-22	-12
8	42	Obesity, goiter	1, 1.5	5	-6	+15
9	22	Sterility, hypometabolism	1.5	4	-22	-12
10	50	Obesity, hypometabolism	1	60	-25	-10
11	36	Hypometabolism	1	24	-34	-20
12	21	Amyloidosis, tubercular enteritis	2, 3	12	-22	-5

\* All but number 12 were females.

have a BMR below -10 per cent; as has been mentioned, this was often the initial justification for instituting thyroid medication. Various symptoms led the physician to determine the BMR in the first place. Most commonly these were obesity, coolness, dryness of the skin, alopecia, somnolence, and menorrhagia. Although some of these symptoms are similar to those found in true myxedema, all cases in which, in retrospect, there is any real possibility of the presence of this disease have been excluded. It cannot be asserted that any syndrome consisting of some of these

symptoms and a low BMR can be at all sharply defined, yet it was our impression that many of these patients clinically had a good deal in common. Thyroid medication was without much beneficial subjective or objective effect in the majority of these subjects. A few found that thyroid medication made it easier to prevent a gain in weight, and continued to use it for this purpose. Certain individuals did feel more energetic while receiving thyroid; this was not necessarily correlated with any change in the BMR. Usually, the patients took thyroid for a period of a few

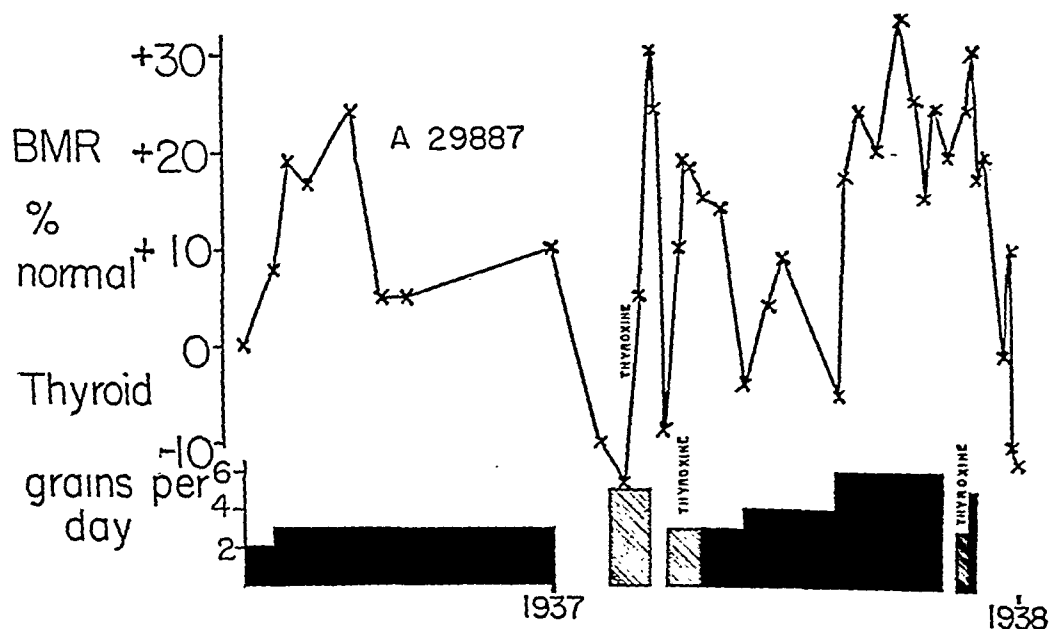


FIG. 5. RISE OF THE BMR WITH SIX BUT NOT WITH FOUR GRAINS OF DRIED THYROID DAILY; POSITIVE RESPONSE TO INTRAVENOUS THYROXINE IN IODOEQUIVALENT AMOUNTS



TABLE III  
Quantitative effects of intravenous thyroxine in various dosages

Case number	Dose	Weeks thyroxine was given	Average initial BMR	BMRs while on thyroxine*	Average increase of BMR on thyroxine	Effect of oral thyroid medication
	<i>mgm. per day</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
A13481	0.64	5	-15	-3, +1, -5	13	No response to 6 grains daily
	1.00	3		+7, +11	24	
	0.29	3		-18, -11	0	
	0.29	3		-9, -19	1	
	0.43	5		+1, +4, -9, -5	7	
85533	0.67	2	-19	-2	17	No response to 5 grains daily
	1.00	3		+9, +10	29	
	0.28	5		-26, -13	0	
	0.43	5		-5, -5, -2, +2		
16186	1.00	5	-23	+3, +6, +8	29	No response to 3 but rise on 5 grains daily
	0.64	4		-7, -2	18	
A29887	0.60	3	-4	+19, +17, +16	21	Adaptation to 4 but sustained rise on 6 grains daily
Table I, 4	0.53	2	-20	-18	2	No response to 3 grains daily
	0.80	3		+4, -2	21	
Table II, 5	0.33	6	-22	-21, -21, -15, -6, -15	4	No response to 2 grains daily

\* After two weeks or more.

months, and then stopped it on their own initiative because it did them little good. On the other hand, no positive deleterious effects were demonstrated. In general, the appearance and reactions of the patients remained unaffected either by the commencement or the cessation of thyroid medication. In this respect, the patients of this group differ sharply from patients with true myxedema, who regularly show both subjective and objective improvement even on small doses of thyroid.

(B) *Persistent elevation of the BMR following long continued administration of dried thyroid*

In the course of this study, a considerable number of subjects were encountered who failed to exhibit any adjustment to prolonged administration of dried thyroid. Occasionally, these patients turned out to have mild myxedema, but in many instances, they derived no subjective or objective benefit from thyroid medication, nor did they have any other stigmata of thyroid deficiency. In Table II are summarized the protocols of twelve patients falling into this group. The number of patients is smaller than the number listed in Table I, mainly because the patients of this second group

often developed unpleasant subjective reactions, such as palpitation, so promptly that they refused to continue thyroid medication for any prolonged period. Only those willing to continue medication for more than two months are included in Table II. The predominance of females is notable. The types of disorders from which these patients suffered is also very similar to those of the patients in Table I.

(C) *Quantitative comparison of the effects of oral thyroid and of intravenous thyroxine in six subjects*

The four subjects presented in Figures 2, 3, 4, and 5 all received courses of intravenous thyroxine and all responded to a sufficient dosage with an increase of the BMR and of the pulse rate. Also, case 4 of Table I and case 5 of Table II received thyroxine. The responses of these six subjects to the different courses of thyroxine, together with a resumé of their responses to oral thyroid, are presented in Table III. In Figure 6, the average increase in the BMR is plotted graphically against the daily dose of thyroxine in each subject. The usual response of subjects with true

myxedema to thyroxine (2), indicated by the dotted line, is included for comparison.

This figure indicates that in the non-myxedematous subjects, more than 0.3 mgm. of thyroxine per day was necessary to raise the BMR, and that 1.0 mgm. of thyroxine daily was necessary to increase the BMR by +25 or +30 per cent. This is about the effect produced in the myxedematous subject by 0.2 or 0.3 mgm. of thyroxine daily, so that evidently all these non-myxedematous subjects were only about one-third as responsive to thyroxine as were those with myxedema. One milligram of thyroxine contains about the same *total* amount of iodine as 4.5 or 5.0 grains of dried thyroid (although only about 0.3 of the iodine in the latter is *thyroxine* iodine). In total iodoequivalent amounts, therefore, thyroxine was much more effective than oral thyroid in four (A13481, 85533, 16186, A29887), and somewhat more effective in two (Table I, case 4 and Table II, case 5), of the subjects tested.<sup>1</sup> At the same

<sup>1</sup> Non-myxedematous subjects do not respond even to the thyroxine content alone of their oral medication as

time, it was much less effective than it would have been in a myxedematous subject. This is another point of difference between the reactions of these patients and of those with myxedema, since the latter respond with the same increase of the BMR to oral thyroid and to intravenous thyroxine when given in iodoequivalent amounts.

#### (D) *Effect of oral thyroid medication on the concentration of iodine of serum*

The little information available on the variations of the serum iodine in these patients is presented in Table IV. The serum iodine concentration of case 16186, while receiving three grains of thyroid daily, was about the same as the concentration

actively as do those with myxedema. About 0.3 of the iodine content of thyroid substance is present as thyroxine iodine. Hence, 6 grains of thyroid, containing about 72 mgm. of total iodine, contains some 22 mgm. of thyroxine iodine, and therefore 33 mgm. of thyroxine. This is a sufficient daily dose of thyroxine to produce a rise of 20 per cent or more in any cases of myxedema; yet, in at least two of the non-myxedematous subjects, 6 grains of oral thyroid daily were without any effect on the BMR.

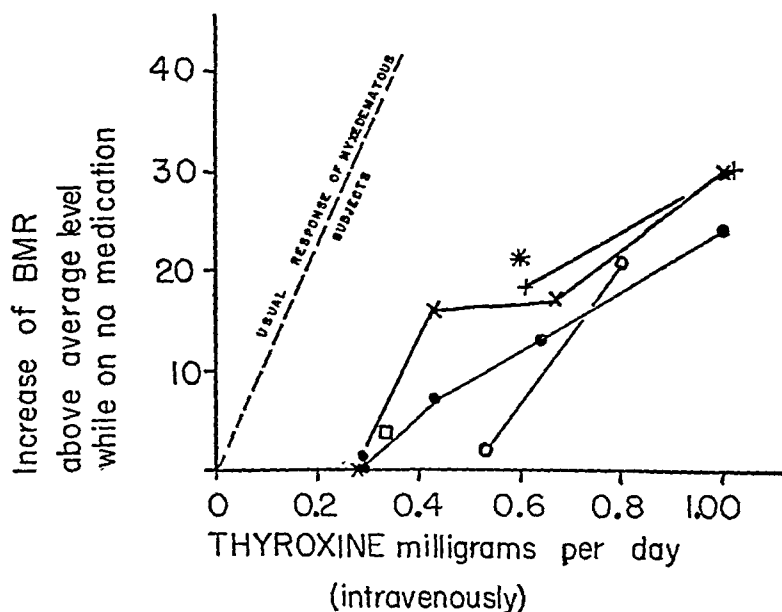


FIG. 6. RESPONSE OF BMR IN SIX NON-MYXEDEMATOUS SUBJECTS TO VARIOUS DOSES OF THYROXINE

Each point corresponds to the average BMR after a course of two weeks or more of intravenous thyroxine in the dose indicated. There is no response unless more than 0.3 milligrams of thyroxine are given daily, and even with larger doses, the response is much less than that usually found in myxedematous subjects.

TABLE IV  
*Serum iodine and thyroid dosage*

Case number	Date	Thyroid medication	BMR	Serum iodine	
				Total	Precipitable
		grains per day	per cent normal	gamma per cent	
16186	December 4, 1940	3	-10	4.0*	
	April 2, 1941	0	-23	4.8	
	December 3, 1941	5	-14	7.4	5.8
	July 22, 1942	5; stopped	+9	5.9	5.9
	July 29, 1942	0	-21	3.4	
	August 19, 1942	0	-23	3.0	
	October 1, 1942	0	-24	4.8	
	November 11, 1942	0	-28	4.8	
A13481	December 16, 1940	0	-15	3.0*	
	December 15, 1941	6	-8	3.0	3.0
A44014	December 23, 1941	5	-8	6.4	5.9
	July 22, 1942	5; stopped	+1	8.1	7.1
	July 30, 1942	0	-10	4.4	3.0
	August 6, 1942	0	-2	3.3	
	August 20, 1942	0; start 2	-20	2.8	
	October 2, 1942	2	-17	5.5	
	November 4, 1942	2	-4	5.5	
	December 2, 1942	4	+8	5.0	
85533	December 10, 1940	0	-17	3.2*	

\* Whole blood iodine divided by 0.6.

while receiving no thyroid, *i.e.*, four to five gamma per cent. On five grains daily, the concentration was maintained at six or seven gamma per cent. This is correlated with the fact that three grains daily did not affect the BMR, while five grains daily always produced a distinct rise. When thyroid extract was abruptly stopped in July of 1942, serum iodine promptly fell to subnormal levels during the next four weeks, then returned spontaneously to a normal level in ten weeks. Changes in the BMR during this time only partially followed those of the serum iodine. Case A13481 exhibited the same rather low concentration of serum iodine while taking six grains of thyroid daily and while taking no medication; this constancy is correlated with the constancy of the BMR in this patient in spite of changing thyroid dosage. No entirely satisfactory control value of the BMR for the serum iodine is available in case A44014. On five grains of thyroid daily, the serum iodine values, 6.4 and 8.1 gamma per cent, were normal or slightly elevated. Cessation of thyroid medication was followed by a precipitous decline of the serum iodine concentration to subnormal levels, as in case 16186. Unfortunately, it is not known whether the iodine concentration would have risen again spontaneously, as it did in case 16186, since the patient resumed thyroid medication. On two grains daily, the serum iodine concentration was 5.5 gamma

per cent, a normal value. Case 85533 had only one, rather low, serum iodine determination while on no medication.

#### DISCUSSION

There are evidently a good many subjects who can tolerate two or three grains of oral thyroid daily, and some who can tolerate six or seven grains, without effect on the BMR. These patients had no other obvious clinical peculiarity in common, save possibly a tendency toward an initially low BMR. The BMR of myxedematous subjects, on the other hand, regularly increases by twenty-five or thirty per cent on two or three grains of thyroid daily, and shows no tendency to decline even after years of thyroid medication (1). Some physiological explanation of this difference in response is clearly needed. At least three interpretations appear possible: (a) thyroid substance may not be properly absorbed from the gastrointestinal tract of these subjects; (b) the tissues of these people are or become peculiarly insensitive to the usual metabolic stimulation by the thyroid hormone; and (c) the hormone is wholly or in part destroyed, stored, or inactivated, soon after it enters the system.

Although the first possibility, that of deficient absorption, cannot absolutely be eliminated, there are weighty arguments against it. These are: (1) the absence of any other defect in absorption or any gastrointestinal lesion in these patients; (2) the fact that no patient with myxedema, spontaneous or following total thyroidectomy, has been found to have any difficulty in responding to oral thyroid (*i.e.* no case relieved by small doses of intravenous thyroxine but not by large doses of oral thyroid extract), which makes it improbable that difficulty in absorbing thyroid extract can be at all common; and (3) the fact that this offers no explanation of the quantitatively diminished response of these patients to thyroxine.

There are at least two important objections to the second type of explanation, which assumes the presence or development of a lowered tissue reactivity. In the first place, if this were true, one would expect no difference between the effects of thyroxine intravenously and those of oral thyroid; in iodoequivalent amounts, they should either produce no effect at all or the same, but diminished,

effect. This is, however, not the case. Thyroxine regularly produced a response while amounts of oral substance containing comparable amounts of iodine produced none. Furthermore, one might expect the serum iodine levels to be abnormally elevated while these insensitive subjects were receiving large amounts of thyroid by mouth; such is, however, not the case.

The third line of explanation—that the hormone is inactivated<sup>2</sup> after absorption of injection—avoids the difficulties inherent in the first two explanations, and is at least compatible with the facts as they are now known. Under this hypothesis it must be assumed that both oral thyroid and intravenously injected thyroxine tend to be inactivated, but that there is an upper limit to the rate at which this can be done and the amount that can be handled. Therefore, oral thyroid, entering the system gradually, would be more readily inactivated than thyroxine, which is suddenly introduced directly into the circulation. This would explain the fact that thyroxine is more effective than oral thyroid in these subjects, and yet is less effective than in myxedematous subjects. It also could explain the fact that the ability to handle thyroid substance is usually not unlimited, and that it may vary a good deal from one individual to another. It is consistent with the relatively low iodine content of the serum in these subjects while they are receiving large doses of thyroid. Speculation that the agent for this inactivation is the thyroid gland itself is naturally suggested by the extraordinary avidity of the gland for iodine (4). If this were the case we should have an explanation for the fact that the development of tolerance is peculiarly absent in subjects with myxedema, since they, presumably, have no functional thyroid tissue. In these non-myxedematous subjects, the drop of the BMR on discontinuing thyroid medication is often much more rapid than that seen in myxedema; this also suggests continuing removal of the effective hormone in subjects possessing a thyroid gland. It is not sufficient to assume that the normal thyroid gland

merely suppresses its own output of hormone when thyroid is given by mouth, as Farquharson and Squires (5) have suggested. Some subjects manage to tolerate at least twice the normal requirements of thyroid hormone, gauged by the amount necessary to maintain the thyroidless subject. There is, however, evidence from the serum iodine figures (Table IV) that some depression of function may take place, which does not immediately disappear when artificial thyroid administration is discontinued.

This hypothesis necessarily requires much more experimental work for its confirmation. A study of the serum iodine levels at intervals after thyroxine injection should prove helpful. Many things at present are difficult to interpret, such as the apparent ability of thyroid medication to keep the weight down, even when the BMR is unaffected. For the present, it is advanced as a tentative hypothesis, more consistent with the facts as they are now known than are the alternative hypotheses, and awaiting further experimental investigation to confirm or disprove it.

#### CONCLUSIONS

(1) Certain non-myxedematous subjects can tolerate as much as six grains of dried thyroid daily for long periods, without effect on the BMR or on the pulse rate. Many others can tolerate as much as three or four grains daily without effect on the BMR.

(2) These subjects respond to thyroxine intravenously, but require much larger doses than do myxedematous subjects to produce a comparable rise in the BMR.

(3) This behavior is very different from the absence of tolerance and the acute sensitivity to thyroid and to thyroxine of the patient with myxedema.

(4) It is suggested as a working hypothesis that this difference of behavior can best be explained by assuming that the non-myxedematous subject possesses the ability, wanting in the patient with myxedema, to inactivate thyroid substance and intravenous thyroxine.

#### PROTOCOLS OF CASES

*A44014.* This white female of English extraction, born in 1899, had a subtotal thyroidectomy for simple goiter in 1924. At various times during the next few years, she

<sup>2</sup> The terms "inactivate" and "inactivation" are used to avoid any unwarranted assumption concerning the particular process involved in the inhibition of the usual role of the hormone. Since the process can continue for months and years, it seems unlikely that storage alone, in the thyroid gland or elsewhere, can afford a complete explanation of the phenomenon.

was given two or three grains of thyroid extract daily because of a tendency toward obesity. Cessation of thyroid administration for months at a time had no effect other than a tendency to gain weight. There were no symptoms suggesting myxedema. She was first seen in this clinic in 1934, at which time her BMR, while taking no dried thyroid, was plus 2 per cent. From 1934 to 1942, she has been treated in this clinic with oral thyroid in doses ranging from three to seven grains daily (Figure 1). For three brief intervals, thyroid was omitted without the development of symptoms suggesting myxedema. In 1936 to 1937, there was an uneventful normal pregnancy, and in 1940, a skin complaint of unknown nature, characterized by subcutaneous tenderness of the arms. This later disappeared spontaneously. In 1941, there was an attack of acute cholecystitis and in 1942, a gall-bladder containing stones was removed surgically without complications. At present (November, 1942), she feels entirely well, and takes small amounts of thyroid in order to counteract her tendency toward obesity.

*A13481.* This woman, born in Italy in 1899, developed typical Graves' disease in 1932. A subtotal thyroidectomy in two stages was performed in June, 1932, following preliminary treatment with iodine. Pathological examination revealed adenomatous hyperplasia of the thyroid gland. Recovery was complete, the BMR one year after operation being minus 3 per cent. In 1933 to 1934, she went through a normal pregnancy. When seen after an absence of one year, in September of 1935, the BMR was found to be minus 15 per cent. There were no subjective or objective disturbances except for obesity and occasional spells of slight vertigo. During the next six years, she was treated at intervals with oral thyroid and with occasional courses of intravenous thyroxine (Figure 2). Her only complaint during this time was moderate obesity. Cessation or resumption of thyroid were without subjective effect in any dose attempted. Pulse rate was also unaffected. She always tended to gain weight rather rapidly whenever thyroid was omitted.

*85533.* This female patient, born in Russia in 1885, was found to have pulmonary tuberculosis, involving the right upper lobe, in 1929. This has remained fairly quiescent ever since, although there have been occasional episodes of blood streaked sputum, without fever, weight loss, or roentgenological evidence of progression; at present (1942), there is probably some bronchiectasis as well as old tuberculous fibrosis. In 1936, the BMR was found to be minus 27 per cent. The metabolism test had been suggested because of headaches and some dryness of the hair. There were no stigmata of myxedema and the serum cholesterol concentration was normal. She has been treated off and on since that time with courses of thyroid and of thyroxine (Figure 3). Thyroid seemed entirely without effect on the BMR or on the pulse rate,

and produced no subjective benefit. It was therefore finally abandoned in 1941, and the patient has since noticed no change of any sort. Thyroxine produced marked tachycardia, rise in the BMR, weight loss, and nervousness.

*16186.* This woman, born in Austria in 1887, developed a migratory arthritis in 1931. This was apparently an acute rheumatism superimposed upon an old chronic hypertrophic arthritis. The acute phase disappeared completely within a few months, but the back and the knee joints have been a little stiff ever since. In 1934, there was a sudden detachment of the right retina, which was believed to be due to myopia. No vascular disease could be detected. In 1936, a BMR, taken because of some nervousness and weight loss, was found to be minus 12 per cent on two occasions. Thyroid, two grains daily, was given for a brief period without subjective improvement, and was therefore discontinued. In 1939, the BMR was again found to be minus 29 and minus 22 per cent on two occasions. There were no stigmata of myxedema; thyroid medication was again given in the hope of improving her general lassitude. This has been continued at intervals until the present (Figure 4); she thinks that she feels a little more energetic while taking thyroid, but does not mind doing without it.

*A429887.* This young girl, born in 1919, has been followed from 1933 until 1942 because of failure to attain a normal stature and because of failure of the menses to appear. General physical status has always been excellent. BMR while taking no thyroid was minus 2 per cent. There were no stigmata of thyroid deficiency. Estrogenic and androgenic activities of the urine were normal. The height (139 cms.) and the menses have been unaffected by courses of antuitrin-S, of thyroid by mouth, and of thyroxine (Figure 5).

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# ELECTROPHORETIC AND CHEMICAL ANALYSIS OF PROTEIN IN NEPHRITIC URINE<sup>1</sup>

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Quantitative chemical determinations of the protein concentration and of the relative proportions of albumin and globulin in the urine of a series of cases of Bright's disease showed that there was a close relationship between high concentrations of globulin on the one hand, and on the other, the rate of progress of renal failure and the collection within glomeruli and tubules of the kidneys of hyaline material which is probably precipitated protein (1). Since these analyses were made by precipitation of protein and macro-Kjeldahl determinations of nitrogen, and since it is said that such precipitation methods are of doubtful value on account of the instability of the biocolloids and their sensitivity to chemical agents (2), it seemed desirable to compare the results of the chemical method with those obtained by the moving boundary electrophoretic method of Tiselius (2).

## THE MATERIAL EXAMINED

Twenty-two specimens of urine were analyzed by both chemical and electrophoretic methods. Twenty-four-hour specimens were collected in clean dry bottles. Either toluene or thymol was used as preservative. Since it has been found that the small change in pH of urines collected under these conditions does not appreciably alter the fractionation and subsequent Kjeldahl determination of nitrogen as previously described (1), no adjustment of pH was made. The urine was filtered through coarse filter paper before analyses of the protein were begun. The concentration of protein in the 22 specimens varied from 0.450 to 3.450 grams per cent (Kjeldahl determinations).

The urine was obtained from 3 cases of progressing nephrotic nephritis, 1 lipoid nephrosis, and 1 case of multiple myeloma with Bence-Jones proteinuria and renal insufficiency (Table I). The kidney disease did not begin in any of the cases as acute hemorrhagic glomerulonephritis. Renal insufficiency progressed at different rates in the 3 cases of nephrotic nephritis.

Patient S, after a 2-year period of stationary renal function and occasional globulinuria of high concentration, during the following year developed frequent globulinuria of high concentration (30 to 40 per cent of the total protein), and the urea clearance fell from 38 and 40

TABLE I  
Total protein in grams per cent, and the relative percentage of globulin, in 22 nephritic urines, as measured by Kjeldahl and electrophoretic methods

Date	Patient	Diagnosis	Total protein (Kjeldahl)	Relative percentage of globulin	
				Kjeldahl	Electrophoretic
			grams per cent		
November 22, 1941	S	Rapidly progressing nephrotic nephritis	0.563	31	37
January 3, 1942	S		0.469	31	36
February 7, 1942	S		0.638	33	36
February 14, 1942	S		0.575	40	36
February 21, 1942	S		0.619	42	38
March 21, 1942	S		0.756	36	31
November 23, 1941	Q	Rapidly progressing nephrotic nephritis	0.956	35	29
December 23, 1941	Q		1.169	38	39
January 4, 1942	Q		0.669	36	38
January 3, 1942	M	Slowly progressing nephrotic nephritis	0.581	21	24
January 24, 1942	M		0.581	31	27
February 7, 1942	M		0.600	31	29
February 14, 1942	M		0.450	31	27
February 21, 1942	M		0.538	29	31
January 26, 1942	A	Lipoid nephrosis	3.031	20	18
February 1, 1942	A		2.225	17	17
February 7, 1942	A		3.450	22	19
February 22, 1942	A		2.225	21	17
September 7, 1941	T	Multiple myeloma and progressive renal insufficiency	0.519	97	100
October 1, 1941	T		0.563	100	100
October 10, 1941	T		0.744	95	99+
January 9, 1942	T		0.475	92	93

to 3 and 3 per cent of normal standard. She died in uremia soon afterwards (blood N.P.N., 184 mgm. per cent).

The urine of patient Q, during a period of 17 months, always contained high concentrations of globulin (30 to 50 per cent of the total protein, usually). At the beginning of this period, the urea clearance was 55 and 48 per cent of normal standard and the blood N.P.N., 30 mgm. per cent. Before she died in uremia, the N.P.N. reached 176 mgm. per cent. Urea clearance was not done.

The renal function of patient M was apparently stationary for 2 years and the urine contained high concentrations of globulin on few occasions. During the following year, high concentrations of globulin (30 to 33

<sup>1</sup> Aided by a grant from the Rockefeller Foundation Fluid Research Fund of the School of Medicine of the Johns Hopkins University.

per cent of the total, generally) began to appear fairly often, and the renal function decreased somewhat (blood N.P.N. rose from 44 to 60 mgm. per cent).

The patient with lipid nephrosis (Table I, patient A) has maintained normal urea clearance and blood N.P.N. for over 2 years, and globulin concentrations in the urine as high as 30 per cent of the total protein have rarely occurred.

The diagnosis of multiple myeloma (patient T) was confirmed by sternal puncture. The patient died with a high degree of renal insufficiency about a year after the onset of symptoms (blood N.P.N., 156 mgm. per cent and urea clearance, 3 and 4 per cent of normal standard 2 months before death).

#### METHODS

*The chemical method.* The chemical method which was used is a modified Howe analysis. Total protein was precipitated by means of trichloroacetic acid; globulin by a 4.05 molar potassium phosphate mixture (3). Nitrogen determinations were made by the macro-Kjeldahl method. The technique employed in precipitation of the urinary proteins, determination of nitrogen, calculation of total protein, albumin, and globulin, and the limitations of these methods have already been described in detail (1). They need not be repeated here.

*The electrophoretic method.* The electrophoretic analyses were performed by the Longworth (4) modification

of the apparatus of Tiselius. Urinary proteins were precipitated by saturation with ammonium sulfate, filtered, redissolved, and dialyzed in running tap water until essentially free of ammonia. They were then dialyzed against a barbital buffer of ionic strength 0.1 and pH 8.5. Under these conditions, the pattern of proteins consists of albumin and the usual 3 globulin components (alpha, beta, and gamma), plus a component not ordinarily seen at a less alkaline pH, which has been denoted as alpha<sub>1</sub> globulin ( $\alpha_1$ ) by Longworth (5). Since it has been found by one of us (6) on fractional salting-out of serum that this component ( $\alpha_1$ ) is even more soluble than electrophoretic "albumin," we have included it in the present urinalyses with the "albumin" fraction, and have considered the sum of the usual  $\alpha$ ,  $\beta$ , and  $\gamma$  fractions as the "true" percentage of globulin.

The electrophoretic measurements were made of the ascending pattern, since irregular refractive effects at the descending beta peaks interfered with the measurements there.

#### RESULTS

The results are shown in Table I. The 2 methods gave values for globulin which agreed closely in their order of magnitude. Neither method gave consistently higher or lower values. In 10 specimens, a slightly higher result was obtained by the

TABLE II  
*Electrophoretic fractionation of 22 nephritic urines*

Date	Patient	Diagnosis	Total protein (Kjeldahl)	Concentrations (per cent of total)					
				Albumin	Globulins				
					Total	Alpha	Beta	Gamma	Fibrinogen
November 22, 1941	S	Rapidly progressing nephrotic nephritis	<i>grams per cent</i> 0.563	63.4	36.6	6.0	11.2	19.4	0
January 3, 1942	S		0.469	64.4	35.6	8.9	8.9	17.8	0
February 7, 1942	S		0.638	64.4	35.6	7.4	10.4	17.8	0
February 14, 1942	S		0.575	64.0	36.0	8.7	9.0	18.3	0
February 21, 1942	S		0.619	62.5	37.5	9.2	9.2	19.1	0
March 21, 1942	S		0.756	69.0	31.0	4.8	10.2	16.0	0
November 23, 1941	Q	Rapidly progressing nephrotic nephritis	0.956	61.5	38.5	4.5	12.0	22.0	0
December 23, 1941	Q		1.169	61.5	38.5	3.4	10.3	24.8	0
January 4, 1942	Q		0.669	62.3	37.7	3.9	9.4	24.4	0
January 3, 1942	M	Slowly progressing nephrotic nephritis	0.581	75.8	24.2	8.3	8.7	7.2	0
January 24, 1942	M		0.581	72.9	27.1	10.0	9.1	8.0	0
February 7, 1942	M		0.600	71.5	28.5	9.1	9.5	9.9	0
February 14, 1942	M		0.450	73.5	26.5	8.7	9.1	8.7	0
February 21, 1942	M		0.538	69.1	30.9	9.1	11.5	10.3	0
January 26, 1942	A	Lipoid nephrosis. Normal renal function	3.031	81.8	18.2	4.8	10.4	3.0	0
February 1, 1942	A		2.225	82.7	17.3	4.6	10.8	1.9	0
February 7, 1942	A		3.450	80.6	19.4	5.6	10.8	3.0	0
February 22, 1942	A		2.225	82.6	17.4	3.5	11.2	2.7	0
September 7, 1941	T	Multiple myeloma. Progressive renal insufficiency	0.519	0	100	0	98	2.0	0
October 1, 1941	T		0.563	0	100	0	100	0	0
October 10, 1941	T		0.744		99+	0	99+		0
January 9, 1942	T		0.475	7.3	92.7	0	91.2	1.5	0

chemical method. In 12 others, electrophoresis gave a slightly higher value. In 3 of the 22 analyses, there was a difference of 5 points, in 1, a difference of 6, in the results obtained by the 2 methods. The difference was less than 5 points in each of the other pairs of analyses.

The results of electrophoretic analyses alone are shown in Table II. No fibrinogen was demonstrable in any of the specimens. The high proportion of globulin in the urine of the patients with rapidly progressing nephrotic nephritis (S and Q, Table II) was due especially to high percentages of gamma globulin. The globulin in the urine of the patient with slowly progressing nephrotic nephritis was about equally divided among the alpha, beta, and gamma fractions (M, Table II). In the urine of patient A, with lipoid nephrosis, the small proportion of gamma globulin is very striking. Most of the protein in the urine of T (multiple myeloma) moved like a beta globulin.

#### DISCUSSION

The close agreement of the results obtained by the 2 methods of analysis indicates that the fractionation and chemical method which we have used is reliable, and, when the concentration of protein in the urine is in the neighborhood of 0.4 grams per cent or more (1), the proportion of albumin and globulin may be determined with a degree of accuracy which is adequate to detect the differences which we have considered to be significant.

In comparing the Kjeldahl and electrophoretic methods, it is important to bear in mind that, so far as we can tell, differences of a few points are of little significance, although if they are expressed in terms of percentage, the variations between the results might seem to be great. Our previous chemical, clinical, and pathological studies of a long series of cases indicated that high or low concentrations of globulin in the urine for prolonged periods are significant in relation to the collection of protein coagula in the kidneys, in glomeruli or tubules or in both. It was found that there is what may be called a critical level for the precipitation of protein in the kidney in those urines with high protein concentrations in which the percentage of globulin lies in the neighborhood of 30 to 35 per cent of the total protein. Due partly to the limitations of the chemical method when applied

to the small quantities of protein found in some urines, it was impossible to evaluate the significance between small differences in the percentage of globulin, such as the difference between 5 and 10 per cent globulin or the difference between 30 and 35 per cent. Since, however, many of the analyses were continued at weekly intervals for many months, it eventually became evident that the urine were below the critical level in relation to precipitation of urinary protein in the kidney, and it became equally evident that concentrations of 30, 35, and 40 per cent were above this level and were associated with the presence of protein coagula in the kidneys.

It is self-evident that errors inherent in a chemical procedure, expressed in absolute percentages of the total, increase as the quantities of material analyzed decrease below the optimum. Therefore, the results of globulin determinations by the chemical and electrophoretic methods are more significant when expressed in terms of their order of magnitude rather than in terms of the absolute percentage variation between the two.<sup>2</sup>

It is well-known that in the presence of chronic proteinuria, the renal insufficiency which develops in some cases is due in part to the accumulation within glomeruli and tubules of varying amounts of hyaline material which resembles precipitated or coagulated serum protein. In others (lipoid nephrosis) exhibiting equal or greater proteinuria, coagula may never form in the kidneys and progressive renal failure may never develop. Evidence obtained from chemical analysis of urinary proteins indicates that the rate of progress of

<sup>2</sup> For example, the specimen "November 22, 1941, S," Table I, was found by both methods to contain a high percentage of globulin, 31 per cent by the chemical method and 37 per cent by electrophoresis. This is a difference of 6 points or an absolute variation of 19 per cent. Specimen "February 22, 1942, A," Table I, was found by both methods to contain a low proportion of globulin, 17 per cent by the electrophoretic and 21 per cent by the chemical method. This smaller variation of 4 points, expressed in percentage, is an apparent difference of 24 per cent. The difference between 35 and 36 is a variation of 2.5 per cent, while a similar difference of one point between 2 and 3 is a variation of 50 per cent. It is therefore obvious that it would be misleading to express the differences between the results of chemical and electrophoretic analysis of urinary protein in terms of percentage.



renal failure of this type is closely associated with the duration and degree of concentration of globulin in urine which contains total protein in high concentration (1). In lipoid nephrosis, and in other cases of chronic nephritis during periods of stationary renal function, high concentrations of globulin were rarely found in the urine. The electrophoretic analyses gave similar results, higher proportions of globulin being found in the more rapidly progressing cases, and low globulin concentration in the case of nephrosis with normal renal function.

The electrophoretic analyses showed in addition that in the urine of the 2 most rapidly progressing cases of renal insufficiency, gamma globulin was especially abundant (Table II, patients S and Q). The gamma globulin was considerably lower in the urine of patient M, and the progress of renal insufficiency has been a good deal slower in this case. The urine of the patient with lipoid nephrosis, and normal renal function for over 2 years, contained low concentration of total globulin by both methods of analysis, and by electrophoresis, gamma globulin was very low (patient A). Longsworth and MacInnes (7) and Luetscher (8) have also found low concentrations of gamma globulin in a few specimens of "nephrotic" urine which they examined electrophoretically. Inasmuch as gamma globulin is the fraction most easily precipitated by any of the usual protein precipitants, it might be expected that the concentration of this protein would be particularly concerned in precipitation within the kidney.

The Bence-Jones protein in the urine of the patient with multiple myeloma and renal insufficiency (Table II, patient T) behaved electrophoretically like a beta globulin. The papers of McFarlane (9), Longsworth, Shedlovsky, and MacInnes (10), Kekwick (11), and Gutman and associates (12), show that increased amounts of protein, with mobilities like beta or gamma globulins, occur in the blood of some cases of multiple myeloma.

The absence of demonstrable amounts of fibrinogen in the urines analyzed electrophoretically is especially interesting because the nephritis did not begin in any of the cases as acute hemorrhagic nephritis. We have already pointed out that in cases of this type (progressing nephrotic nephritis), most of the protein precipitated in the kid-

ney does not resemble fibrin microscopically; and, in the glomeruli, it collects in an intercapillary position which is different from the location of the fibrin clots which can be readily identified in Bowman's spaces in some cases of acute hemorrhagic nephritis (1).

#### SUMMARY

The relative percentage of globulin in samples of a series of 24-hour specimens of nephritic urine was determined by a chemical method and by the electrophoretic moving boundary method of Tiselius. The concentration of total protein in the specimens varied from 0.450 to 3.45 grams per cent. The results obtained by the 2 methods agreed closely.

In the present small series of electrophoretic determinations, the concentration of gamma globulin in particular was high in urine of patients whose renal insufficiency was progressing rapidly, and the same fraction was very low in the urine of a patient with chronic lipoid nephrosis and normal renal function.

The absence of demonstrable quantities of fibrinogen in the urine of the patients with progressing nephrotic nephritis tends to confirm our previous conclusion that the hyaline materials which collect in glomeruli and tubules of these cases are probably derived from globulins other than fibrinogen.

The Bence-Jones protein in the urine of a patient with multiple myeloma and renal insufficiency behaved electrophoretically like a beta globulin.

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